REMARKS

Amendments to the Claims

Claims 12, 14-15, 26 and 27 have been canceled.

Claims 1, 3-5, 7-8, 11, 24 and 28-32 have been amended.

Claims 1, 3, 4, 5, 7, 8 and 11 have been amended to recite "a method of treating inflammation associated with viral infection...." Support is found in the specification, for example at page 10, lines 16-25; page 16, lines 9-15; page 57, line 17 to page 59, line 4, particularly page 58, lines 10-14. In addition, support is found in priority application US Serial No. 07/670,827, filed March 18, 1991, at page 10, line 22 to page 11, line 9; and page 39, line 24 to page 40, line 9. This priority application is incorporated in the subject application by reference on page 1, lines 4-21.

Claim 24 has been amended to recite "the method of Claim 23 wherein said single or divided dose is one selected from 0.5, 0.9, 1, 1.1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 mg/kg per day on at least one of day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 or at least one of week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20." Support is found in the specification, for example, at page 60, lines 15-24. In addition, support is found in the specification of priority application US Serial No. 07/943,852, filed September 11, 1992, for example, at page 42, lines 5-18.

Claim 28 has been amended to recite "the method of Claim 1, wherein said human suffers from alcohol-induced hepatitis." Support for this new claim is found in the specification, for example, on page 58, lines 5-9; and page 59, line 11. In addition, support is found in priority application 07/670,827, filed March 18, 1991, on page 10, line 22 to page 11, line 4 and page 39, line 20 to page 40, line 9.

Claims 29-32 have been amended to correct typographical errors to clarify dependency.

No new matter has been added by the amendments. Therefore, entry of the amendments into the application is respectfully requested.

Examiner Interview

Applicants wish to thank the Examiner for meeting with the undersigned to discuss the application, and for providing helpful comments.

Amendment to the Specification

The title and abstract have been amended to recite "inflammation associated with viral infection" to be more descriptive of the claims, as amended. Support is found in the specification, for example at page 10, lines 16-25; page 16, lines 9-15; page 57, line 17 to page 59, line 4, particularly page 58, lines 10-14. In addition, support is found in priority application US Serial No. 07/670,827, filed March 18, 1991, at page 10, line 22 to page 11, line 9; and page 39, line 24 to page 40, line 9. This priority application is incorporated in the subject application by reference on page 1, lines 4-21.

No new matter has been added by the amendments. Therefore, entry of the amendments into the application is respectfully requested.

Priority

A. Priority of Claims 1, 3-5, 11, 21, 23-24 and 28-32

The Examiner states on page 2 of the Office Action dated March 24, 2006 that "the recitation of 'TNF-α-mediated viral infection' is not readily apparent either in the pending or the priority application." Claims 1, 3-5 and 11, as amended, recite "a method of treating inflammation associated with viral infection in a human." Claims 21, 23 and 28-32 are dependent on these claims and therefore contain the same limitation. As discussed below, the claims, particularly as amended, are entitled to claim the benefit of priority application USSN 07/670,827, filed March 18, 1991.

The priority application USSN 07/670,827, discloses at page 39, line 20 through page 40, line 3 that "[t]he antibodies, fragments, and derivatives of the present invention are useful for treating a subject having a disease or condition associated with levels of a substance reactive with an anti-TNF antibody, in particular TNF, in excess of the levels found in a normal healthy subject. Such diseases include... bacterial, viral and fungal infections." Additionally, support for the use of anti-TNF antibodies to treat inflammation associated with viral infection is found in

this priority application at page 3, lines 13 through 26, which discloses that "TNF is noted for its pro-inflammatory actions which result in tissue injury," and that "[r]ecent evidence implicates TNF in the pathogenesis of many infections." Further support is found in this priority application at page 10, line 22 through page 11, line 2; and page 15, lines 9 through 13. Because this priority application teaches the treatment of TNF-α-mediated inflammation by administration of anti-TNF antibodies and TNF-α-mediated inflammation is associated with viral infection, the priority application discloses the method of treating inflammation associated with viral infection by administration of anti-TNF antibodies. Therefore, the claims as amended are entitled to claim the benefit of priority application USSN 07/670,827, filed March 18, 1991.

As discussed above, Claim 24 is entitled to is entitled to claim the benefit of priority application USSN 07/943,852, September 11, 1992.

B. Priority of Claims 7-10

The Examiner states on page 3 of the Office Action dated March 24, 2006 that "wherein said anti-TNF chimeric antibody comprises a non-human variable region comprising an amino acid sequence selected from the group consisting of SEQ ID NOS: 2, 3, 4 and/or 5" would appear, at best, to receive a priority date back to USSN 08/192,093, filed 2/4/94.

Claims 7-8 recite "[a] method of treating inflammation associated with viral infection..." Claims 9-10 are dependent on these claims and therefore contain the same limitation. As discussed above, the method of treating inflammation associated with viral infection is entitled to claim the benefit of priority application USSN 07/670,827, filed March 18, 1991.

Claims 7 and 8 as amended recite that the non-human variable region comprises an amino acid sequence selected from the group consisting of SEQ ID NO.: 3 and SEQ ID NO.: 5. Claims 9 and 10 as amended recite that the non-human variable region comprises a polypeptide encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NO.: 2 and SEQ ID NO.: 4. As discussed below, due to the biological deposit for the A2 antibody (designation c134A) with American Type Culture collection (ATCC) under the Budapest Treaty, which was deposited on September 22, 2005, Claims 7-10 are entitled to claim the benefit of priority application USSN application 07/670,827 (filed March 18, 1991).

The Federal Circuit has held that reference in the specification to a deposit in a public depository, which makes its contents accessible to the public, constitutes an adequate description of the deposited material sufficient to comply with the written description requirement of section 112. *Enzo Biochem v. Gen-Probe* , 323 F.3d 956, 966 (Fed. Cir. 2002). The *Enzo* court reasoned that a person of skill in the art, reading the accession numbers in the patent specification, can obtain the claimed sequences from the ATCC depository by following the appropriate techniques to excise the nucleotide sequences from the deposited organisms containing those sequences. Thus, the court concluded that "reference in the specification to deposits of nucleotide sequences describe those sequences sufficiently to the public for purposes of meeting the written description requirement." *Id.* at 965-966.

Based on the teachings of the specification, one of ordinary skill in the art would have concluded that Applicants were in possession of the claimed invention. Therefore, the priority application USSN 07/670,827 (filed March 18, 1991) provides sufficient written description for Applicants' claims and Applicants are entitled to claim the benefit of it. This priority application has been properly referenced on page 1 of the specification in compliance with 35 U.S.C. § 120.

Even if the Examiner were to disagree, Claims 7-10 are at least entitled to claim the benefit of priority application USSN 07/853,606, filed March 18, 1992. The priority application provides sufficient written description and enablement for Claims 7-10 (see, for example, the specification at page 12, lines 20-23; Figures 17A-B; page 24, lines 5-17). Please note that the SEQ ID NO. identifiers were later amended to correct an inadvertent error in the specification to clarify that Figure 17B (renumbered Figure 16B after amendment), is a nucleic acid sequence (SEQ ID NO.: 4) and corresponding amino acid sequence (SEQ ID NO.: 5) of the heavy chain variable region of the cA2 monoclonal antibody (see, for example, page 2 of the Amendment filed in US Serial No.: 08/324,799 on March 14, 1997). The nucleic acid sequence (SEQ ID NO: 2) and corresponding amino acid sequence (SEQ ID NO:3) of the light chain are also disclosed. Thus, Claims 7-10 are at least entitled to claim the benefit of priority application USSN 07/853,606 (filed March 18, 1992), as discussed above.

Rejection of Claims 1, 3-5, 7-12, 14-15, 21, 23-24 and 26-32 Under 35 U.S.C. § 112, First Paragraph

Claims 1, 3-5, 7-12, 14-15, 21, 23-24 and 26-32 have been rejected under 35 U.S.C. § 112, first paragraph, for lack of written description. The Examiner states on page 4 of the office action dated March 24, 2006 that the recitation of "TNF-α-mediated viral infection" is not readily apparent in the pending application.

As noted above, in order to expedite prosecution, Claims 12, 14-15 and 26-27 have been canceled. Claims 1, 3-5, 7-8 and 11 have been amended to recite "a method of treating inflammation associated with viral infection" in place of "a method of treating TNFα-mediated viral infection." Claims 9-10, 21, 23-24 and 28-32 are dependent on these claims and therefore contain the same limitation. As noted above, the specifications of both this application and the priority application US Serial No. 07/670,827, filed March 18, 1991, provide sufficient written description for the claims, particularly as amended. The specification discloses that inflammation is mediated by TNF-α, and that elevated levels of TNF-α are associated with viral infection. Therefore, the specification discloses the treatment of inflammation associated with viral infection by the administration of an anti-TNF antibody. As discussed above, Claim 24 is entitled to is entitled to claim the benefit of priority application USSN 07/943,852, September 11, 1992.

The Examiner further states on page 4 that "neither the priority applications nor the instant application provides a sufficient description of a representative number of species to represent the entire genus of 'TNF-α-mediated viral infections', as currently claimed." (emphasis omitted). As noted above, the claims as amended refer to "viral infection" rather than "TNF-α-mediated viral infection." The genus of "viral infections" is specifically disclosed in the specification, for example, at page 10, lines 21 through 25; page 16, line 10; and page 58, lines 5 through 9. Additionally, the genus of "viral infections" is specifically disclosed in the priority application US Serial No. 07/670,827, filed March 18, 1991, for example, at page 3, line 21 through page 4, line 2; page 10, line 22 through page 11, line 2; and page 39, line 20 through page 40, line 3. Therefore, both the current application and the priority application provide sufficient written description for the treatment of inflammation associated with a viral infection.

Reconsideration and withdrawal of the rejection are respectfully requested.

Rejection of Claims 1, 3-5, 7-12, 14-15, 21, 23-24 and 26-32 Under 35 U.S.C. § 112, First Paragraph

Claims 1, 3-5, 7-12, 14-15, 21, 23-24 and 26-32 are rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Applicants respectfully disagree. The standard for determining whether the specification meets the enablement requirement is whether the experimentation needed to practice the invention is undue. MPEP §2164.01 (8th Edition, Revised August 2005). There are many factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is "undue." These factors include, but are not limited to: the breadth of the claims; the nature of the invention; the state of the prior art; the level of one of ordinary skill; the level of predictability in the art; the amount of direction provided by the inventor; the existence of working examples; and the quantity of experimentation needed to make or use the invention based on the content of the disclosure. MPEP §2164.01(a); *In re Wands,* 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). It is improper to conclude that a disclosure is not enabling based on an analysis of only one of the above factors while ignoring one or more of the others. MPEP §2164.01(a). The Examiner's analysis must consider all of the evidence related to each of these factors, and any conclusion of nonenablement must be based on the evidence as a whole. *Id.* Further, it is well established that "[e]nablement is not precluded by the necessity for some experimentation such as routine screening." *In re Wands,* 8 U.S.P.Q.2d at 1404. "[A] considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *Id.*

On page 5 of the Office Action dated March 24, 2006, the Examiner states that "pharmaceutical therapies in the absence of in vivo clinical data are unpredictable for the following reasons; (1) the protein may be inactivated before producing an effect, i.e. such as proteolytic degradation, immunological inactivation or due to an inherently short half-life of the protein; (2) the protein may not reach the target area because, i.e. the protein may not be able to cross the mucosa or the protein may be absorbed by fluids, cells and tissues where the protein has no effect; and (3) other functional properties, known or unknown, may make the protein

unsuitable for in vivo therapeutic use, i.e. such as adverse side effects prohibitive to the use of such treatment."

In regard to the Examiner's statement regarding proteolytic inactivation, Applicants note that A2-specific monoclonal antibodies have long half-lives (for example, REMICADE® infliximab has a serum half-life of 9.5 days and still detectable in serum 8 weeks after infusion) (see Exhibit A, Cornillie *et al.*, "Infliximab Induces Potent Anti-inflammatory and Local Immunomodulatory Activity but no Systemic Immune Suppression in Patients with Crohn's Disease," *Aliment. Pharmacol. Ther., 15*: 463-473, at 463 (2001)). In fact, monoclonal antibodies by their nature have long half-lives. A2-specific monoclonal anti-TNFα antibodies are generally administered intravenously or subcutaneously. They are not subject to proteolytic degradation, which may occur with oral administration of certain proteins. In regard to the Examiner's statement regarding reaching the target area, Applicants note that the A2-specific antibodies do cross the mucosa as seen in clinical trials with Crohn's disease (see Exhibit A). A2-specific antibodies, such as REMICADE® infliximab, are effective in treating inflammation associated with a viral infection. Further, in regard to the Examiner's statement regarding suitability of A2-specific antibodies for *in vivo* therapeutic use, Applicants note that A2-specific antibodies, such as REMICADE® infliximab, have been used for over a decade to treat effectively other diseases associated with excess levels of TNFα such as Crohn's disease, establishing that these antibodies are safe and effective for *in vivo* therapeutic use (see Exhibit A).

The Examiner further states that "given that TNF-α can have potent anti-viral effects, there is insufficient direction and guidance as to how to 'treat TNF-α mediated viral infections' by administering 'a TNF-α-inhibiting amount of a TNF-α antibody,' which would block the anti-viral effects of TNF-α and, in turn, either exacerbate viral infections or not treat viral infections." (emphasis omitted). Applicants disagree with this assertion. As discussed above, the claims have been amended to recite "a method of treating inflammation associated with a viral infection." Further, as discussed in the enclosed references (Exhibits B-C), TNF-α antagonists, including the antibodies of the present invention, have been used safely and effectively to treat inflammation associated with viral infection. Expert testimony and articles published after the filing date, can show that an invention was properly enabled when this evidence shows the state of knowledge in

the art as of the filing date. *Gould v. Quigg*, 822 F.2d 1074, 1077, 3 USPQ2d 1302, 1304 (Fed. Cir. 1987).

The Examiner states in the Office Action on page 6 that "[t]here is insufficient direction and guidance as to how choose which viral infections are amenable to treatment with anti-TNF-α antibodies. The claims appear to run contrary to the antiviral effects of TNF-α." The claims as amended are now directed to a method of treating inflammation associated with a viral infection. Although TNFα expressed at normal levels may have some antiviral protective effect, it is known in the art that excess levels of TNFα lead to inflammation and tissue damage.[1] One of skill in the art would know that inflammation associated with viral infection occurs in connection with the harmful over-production of TNFα, not with the potential protective effects of low levels of TNFα. Therefore, one of skill in the art would know how to choose when inflammation associated with a viral infection would be amenable to treatment with anti-TNFα antibodies.

Reconsideration and withdrawal of the rejection are respectfully requested.

Rejection of Claims 1, 3-5, 7-12, 14-15, 21, 23-24 and 26-32 Under 35 U.S.C. § 112, Second Paragraph

Claims 1, 3-5, 7-12, 14-15, 21, 23-24 and 26-32 are rejected under 35 U.S.C. § 112, second paragraph as being indefinite in the recitation of "TNFα-mediated viral infection." The Examiner states on page 7 of the Office Action dated March 24, 2006 that "the metes and bounds of said 'TNFα-mediated viral infection' is ill-defined and ambiguous." The Examiner further states that viruses cause viral infection, not cytokines such as TNFα.

As discussed above, in order to further prosecution, Claims 12, 14-15 and 26-27 have been canceled and Claims 1, 3-5, 7-8, and 11 have been amended to recite "inflammation associated with viral infection" rather than "TNFα-mediated viral infection." Claims 9-10, 21, 23-24 and 28-32 are dependent on these claims and therefore contain the same limitation. These claims, as amended, are definite because the term "viral infection" is a term of general

[1] See, e.g., (Exhibit B) Beutler, B. and Grau, E.G., "Tumor Necrosis Factor in the Pathogenesis of Infectious Diseases," *Crit Care Med* 21(10 Suppl): S423-S535 (Oct. 1993) at p. S425; and (Exhibit C) Peper, R.L. and Van Campen, H. 2005, "Tumor Necrosis Factor as a mediator of Inflammation in Influenza A Viral Pneumonia," *Microbial Pathogenesis*, 19: 175-183 (1995).

knowledge and a person of ordinary skill in the art would understand the metes and bounds of treating inflammation associated with that viral infection.

Reconsideration and withdrawal of the rejection are respectfully requested.

Rejection of Claims 1, 3-5, 7-12, 14-15, 21, 23-24 and 26-32 Under 35 U.S.C. § 102(b)

Claims 1, 3-5, 7-12, 14-15, 21, 23-24 and 26-32 have been rejected under 35 U.S.C. § 102(b) as being anticipated by Le *et al.*, U.S. Patent No. 5,919,452.

Le *et al.* (filed February 4, 1994, published July 6, 1999) is not prior art under 35 U.S.C. § 102(b) because it was not published more than one year prior to the filing date of the priority application. As discussed above in the section regarding priority, Claims 12, 14-15 and 26-27 have been canceled and Claims 1, 3-5, 7-11, 21, 23 and 28-32 are entitled to claim the benefit of priority application US Serial No. 07/670,827, filed March 18, 1991. As discussed above, Claim 24 is entitled to is entitled to claim the benefit of priority application USSN 07/943,852, September 11, 1992.

Hence, Applicants' patent Le *et al.*, U.S. Patent No. 5,919,452 is not prior art. Thus, reconsideration and withdrawal of the rejection are respectfully requested.

CONCLUSION

In view of the above amendments and remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned.

Respectfully submitted,

HAMILTON, BROOK, SMITH & REYNOLDS, P.C.

By Deirdre E. Sanders

Deirdre E. Sanders
Registration No. 42,122
Telephone: (978) 341-0036
Facsimile: (978) 341-0136

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Infliximab induces potent anti-inflammatory and local immunomodulatory activity but no systemic immune suppression in patients with Crohn's disease

F. CORNILLIE*, D. SHEALY*, G. D'HAENS†, K. GEBOES‡, G. VAN ASSCHE†, J. CEUPPENS§, C. WAGNER*, T. SCHAIBLE*, S. E. PLEVY¶, S. R. TARGAN¶ & P. RUTGEERTS†

**Centocor Inc., Malvern, PA; the University Hospitals KU Leuven, Departments of †Gastroenterology, ‡Pathology and §Immunology, Leuven, Belgium; and ¶Cedars-Sinai Inflammatory Bowel Disease Center, Los Angeles, CA, USA*

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SUMMARY

Background: Anti-TNFα therapy with infliximab is effective for Crohn's disease. Infliximab neutralizes the biological activities of TNFα, a cytokine involved in host-defence against certain infections.

Aim: To evaluate the effects of infliximab on the gut and peripheral immune system functions.

Methods: Biopsies and blood samples from three clinical trials of infliximab in Crohn's disease were analysed. Pharmacokinetics, changes in leucocyte counts and T cell subsets, T cell function, and cytokine profiles of lamina propria mononuclear cells (LPMC) and peripheral blood mononuclear cells (PBMC) were analysed.

Results: Infliximab has a serum half-life of 9.5 days and is still detectable in serum 8 weeks after infusion. Leucocyte counts showed consistent changes from baseline toward normal values after therapy. Monocytes and lymphocytes were modestly increased, while neutrophils were decreased 4 weeks after treatment. Lymphocyte subsets and T cell proliferative responses were not altered after therapy. The proportion of PBMCs capable of producing IFNγ and TNFα did not change, while Th1 cytokine production by stimulated LPMC was decreased after infliximab therapy.

Conclusion: The clinical efficacy of infliximab is based on local anti-inflammatory and immunomodulatory effects in the bowel mucosa, without generalized suppression of systemic immune functions in Crohn's disease patients.

INTRODUCTION

Infliximab (chimeric anti-human TNFα, Remicade) induces profound and durable responses in a large number of patients with moderate to severe and fistulizing Crohn's disease.[1, 2] The long duration of response suggests that infliximab's mechanism of action may be more complex than simply neutralizing soluble TNFα.

Local effects of infliximab on the inflamed bowel mucosa have been observed. Thus, the number of TNFα expressing cells is reduced 4 weeks after treatment with infliximab in Crohn's colitis. CD4+ and CD8+ cells (the major T cell subtypes), and CD68+ monocytes and macrophages, all important producers of TNFα, are reduced by > 50% in the colonic lamina propria of infliximab-treated patients but not in placebo-treated patients.[3] The number of IFNγ and TNFα-producing mononuclear cells isolated from the lamina propria in patients with Crohn's disease is decreased after treatment with infliximab.[4] Infliximab reduces the expression of the adhesion molecules ICAM-1 and LFA-1.[3] Together with histological repair, remarkable endoscopic healing has been observed, and a correlation between endoscopic healing and reduction of disease

Correspondence to: Dr F. Cornillie, Centocor, 62 Interleuvenlaan, 3001 Heverlee, Belgium.
E-mail: cornillief@centocor.com

activity has been demonstrated after infliximab therapy.[5] The profound clinical, biological, histological, and endoscopic effects of infliximab in patients with Crohn's disease may be explained by the rapid and effective neutralization of TNFα by infliximab, although lysis of activated macrophages which display cell surface transmembrane TNFα may also be important.

The impact of infliximab on systemic immunity is not clear. While TNFα can influence the inflammatory process and modulate the immune response to specific pathogens, the primary defect reported in mice deficient in TNFα is altered cell organization in secondary lymphoid tissues.[6] Although mild infections such as upper respiratory tract infections are increased in infliximab-treated patients, opportunistic infections were not observed.[7] Therefore, it is possible that infliximab exerts significant effects on the pathologic mucosal immune response in Crohn's disease, while systemic immune responses remain intact.

This study reviews data collected during several clinical studies of infliximab in Crohn's disease and aims at evaluating the effect of infliximab on the local (bowel mucosa) and systemic immune functions in treated patients.

MATERIALS AND METHODS

Clinical studies

This paper describes findings from previous clinical trials of infliximab in Crohn's disease.

The T11 study included 20 patients with active Crohn's disease. This was an open-label, dose-ranging study which enrolled five patients per dose group receiving a single infusion of 1, 5, 10 or 20 mg/kg of infliximab. Blood samples were obtained before infliximab treatment and at 1, 2, 4, 24 and 72 h and 1, 2, 4, 8 and 12 weeks following infliximab treatment.

The T16 study was a double-blind, placebo-controlled trial that included 108 patients with moderate to severe, active and refractory Crohn's disease. Patients were treated with a single infliximab infusion of 5 mg/kg ($n = 27$), 10 mg/kg ($n = 28$), 20 mg/kg ($n = 28$) or placebo ($n = 25$). Serum samples were obtained prior to and after infusion of infliximab at 1, 2 and 4 h and at 2, 4, 8 and 12 weeks.

The T20 study was a double-blind, placebo-controlled trial performed in 94 patients with fistulizing Crohn's disease not responding to conventional therapy. Three intravenous infusions of 5 mg/kg ($n = 31$) or 10 mg/kg ($n = 32$) infliximab or placebo ($n = 31$) were given at weeks 0, 2 and 6. Blood samples were collected prior to and 1 h after each infusion, and at weeks 10, 14 and 18 after the first infliximab treatment.

Serum concentrations of infliximab were measured in T16 and T20. TNFα was measured in serum samples collected in T11. Both IL-6 and C-reactive protein (CRP) were evaluated in serum samples from T16. Peripheral white blood cell counts (WBC) were performed in both T16 and T20. The proliferative response of peripheral blood mononuclear cells (PBMC) was analysed in a subset of patients from T16 and T20. Cytokine production by PBMC was evaluated in a subset of patients in T16, and in lamina propria mononuclear cells (LPMC) isolated from a subset of patients in T11 and T16. Pre- and post-treatment samples were compared in individual patients, and diseased tissue was compared with normal tissue from uninvolved areas in the same patient.

Serum assays

Serum concentrations of infliximab were determined using a monoclonal antibody-based enzyme immunoassay (detection limit 0.1 μg/mL). Serum concentrations of TNFα were measured using a commercially available immunoassay kit (detection limit < 3 pg/mL; Biosource, Belgium).

CRP serum concentrations were measured by rate nephelometry (detection limit 0.1 mg/dL). Interleukin (IL)-6 concentrations were determined by enzyme immunoassay (detection limit 0.1 pg/mL; R&D Systems).

Peripheral white blood cell counts (WBC) and lymphocyte subset analysis

Analyses of WBC counts were incorporated into the haematology evaluations that were routinely conducted before and after infliximab treatment in each clinical trial. Lymphocyte subset measurements were performed in patients before and 6 weeks after infliximab infusion. Fifty microlitres of heparinized blood were incubated at 4 °C with an appropriate dilution of a fluorescein isothiocyanante (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibody (Becton-Dickinson, CA, USA) specific for lymphocyte surface markers. After haemolysis of erythrocytes (twice-repeated addition of

NH_4Cl, pH = 7.3), the cells were washed twice in PBS and re-suspended in 1 mL of 1% paraformaldehyde. Combinations of monoclonal antibodies were selected in order to determine the following lymphocyte subsets:

1 T lymphocytes: CD3+ and CD5+ total T cells, α/β+ and γ/δ+ T cells, CD4+ helper/inducer and CD8+ suppressor/cytotoxic T cells, CD8+/CD28+ (CD28 binds to B7 on APCs), CD3+/CD56+ and CD57+ (CD56 and CD57 are markers of NK cell activity on non-T cells, and for long-term memory on T cells), CD3+/CD25+ (CD25 is the IL-2 receptor α-chain) T cells and total HLA-DR+, CD3+/HLA-DR+, CD4+/HLA-DR+, CD8+/HLA-DR+.
2 B lymphocytes: CD19+ and CD19+/CD5+ B cells.
3 Non-T non-B lymphocytes: total, CD56+/CD16+, CD57+, CD8+.

Monocytes were counted by immunofluorescence with PE labelled anti-CD14. Two-colour immunofluorescence analysis was carried out on a Becton–Dickinson fluorescence-activated cell sorter (FACS). Lymphocytes were gated on the basis of forward and 90° light scatter, and 2000–5000 cells were counted in each analysis. Cells were scored positive or negative according to their fluorescence, with reference to that of control samples, incubated with FITC- and PE-conjugated mouse IgG. The number of positive cells was expressed as a percentage of the total lymphocyte count.

Isolation and in vitro *stimulation of Peripheral Blood Mononuclear Cell (PBMC) fractions*

PBMC were isolated from 30 mL of heparinized blood drawn prior to and 4 weeks after infusion of infliximab on Ficoll-Hypaque (density 1.077) gradients (Pharmacia, Uppsala, Sweden). After three washings in Hanks' balanced salt solution, the cells were re-suspended in RPMI 1640 culture medium (Gibco, Paisley, UK) supplemented with 2 mM L-glutamine, penicillin (100 U/mL), streptomycin (100 μg/mL), and 5% normal human serum. To investigate the proliferative response of PBMC to antigenic and mitogenic stimuli, the cells (1×10^5) were cultured in 96-well round-bottomed plates (Costar Europe, Badhoevedorp, Netherlands) with a variety of soluble antigens and mitogens. The following antigens were used: *Candida albicans* (10 μg/mL, Haarlem Allergenen Laboratorium, Netherlands), varidase (100 U/mL, Lederle, Cyanamid Benelux, Brussels, Belgium), tuberculin (20 U, Statens Serum Institute, Copenhagen, Denmark) and heat shock protein-65 (HSP-65, 5 and 10 μg, a gift from J. Van Embden, Bilthoven, Netherlands). The following mitogens were used: pokeweed mitogen (PWM, 0.5 μg/mL, Sigma, St Louis, MO, USA), anti-CD3 (5 μg/mL, polyclonal stimulator UCTH-1, a gift from P. Beverley, Imperial College, London, UK), IL-2 (50 U/mL, Boehringer Mannheim), anti-CD3 plus IL-2 (50 U/mL), anti-CD3 plus phorbol myristate acetate (PMA, 25 ng/mL, Calbiochem Novabiochem), and PMA (25 ng/mL) plus ionomycin (Ca-ionophore 1 μg/mL, Calbiochem Novabiochem).

Cultures were incubated in quadruplicate at 37 °C in a 5% CO_2 atmosphere for 6 days, and 8 h after a 1 μCi (^{3}H)thymidine pulse (2 Ci/mmol; Amersham, Buckinghamshire, UK) cells were harvested and processed for the determination of (^{3}H)thymidine incorporation. Incorporation by cells cultured in medium alone was substracted from the values for antigen and mitogen stimulated incorporation.

Cytokine production by PBMC in vitro

In vitro release of TNFα, IFNγ and IL-10 by PBMC stimulated with concanavalin A (Sigma, St Louis, MO) and PMA (Sigma) were studied before and at 1, 2, 3 and 4 months after infliximab treatment, as previously described.[4]

Cytokine production by LPMC in vitro

Mononuclear cells from bowel lamina propria were isolated before and 1, 2, 3 and 4 months after infliximab treatment from biopsies of inflamed areas, as well as from uninvolved (visually normal) areas of colon. The number of cells stimulated to secrete these test cytokines was quantified by reversed enzyme linked immunospot assay (relispot assay)[4] and compared to matched, unstimulated cells. TNFα, IFNγ and IL-10 release *in vitro* by unstimulated (control) and anti-CD2 and anti-CD28 stimulated LPMC was quantified.

RESULTS

Serum infliximab concentrations

A dose-dependent maximum serum concentration (C_{max}) of infliximab was observed after a single infusion of 5 mg/kg, 10 mg/kg or 20 mg/kg infliximab (Figure 1). At the 5 mg/kg recommended dose the median C_{max} was 118 μg/mL, and the terminal half-life

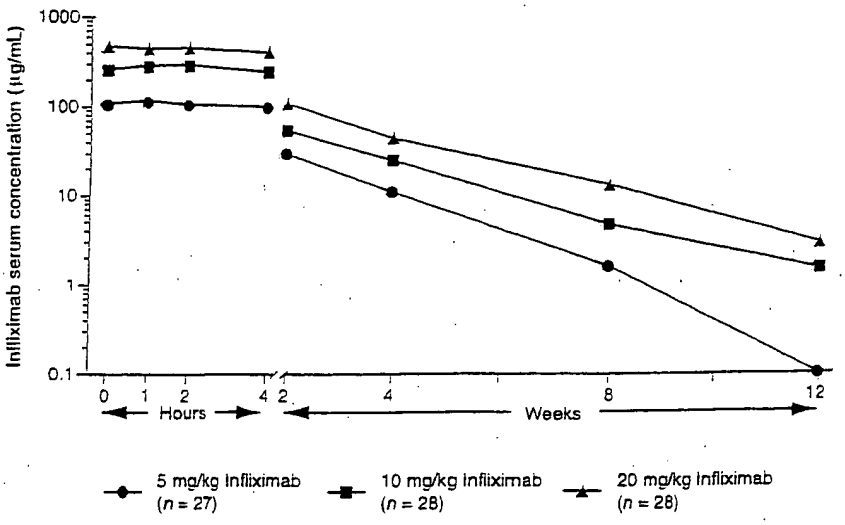

Figure 1. Profile of serum infliximab concentration vs. time after a single infusion of 5, 10 or 20 mg/kg of infliximab in Crohn's disease patients. Each point represents the median serum concentration. The first set of results after the break in the *x*-axis was obtained 2 weeks after the end of the infusion.

of infliximab was 9.5 days. Clearance of infliximab from the circulation was 9.8 mL/h for the 5 mg/kg dose and was similar for the other doses studied. At week 12, infliximab was no longer detectable (median concentration < 0.1 μg/mL) in the 5 mg/kg dose group.

Further analysis showed that there was no difference in the serum levels of infliximab between clinical responders (defined as a ≥ 70 point reduction in the Crohn's disease activity index 4 weeks after infliximab treatment) and non-responders. Median serum levels were 99.5 μg/mL and 118 μg/mL at 2 h post-infusion of 5 mg/kg infliximab, 28.6 μg/mL and 29.7 μg/mL at 2 weeks, and 10.9 μg/mL and 10.6 μg/mL at 4 weeks in responders and non-responders, respectively. Therefore, the difference in response to treatment cannot be explained by a difference in pharmacokinetics.

Re-administration of infliximab at weeks 2 and 6 (as in the T20 study of fistulizing patients) restored the initially achieved serum infliximab concentrations. After the first, second and third infusions of infliximab, the median serum concentrations were 158 μg/mL, 195 μg/mL and 178 μg/mL, respectively, for the 5 mg/kg dose group (Figure 2).

Serum TNFα

Assays of TNFα in serum revealed low levels (5–25 pg/mL) of TNFα at baseline, followed by a rise in TNFα from 4 to 72 h. Peak concentrations of TNFα were detected at 72 h, to 2 weeks, which then decreased to baseline by week 12 (Figure 3). This immune reactivity in the TNFα assay detected after infusion of infliximab was shown to be TNFα-complexed with infliximab.[8] This was confirmed by testing the 72 h serum samples in a WEHI cell cytotoxicity assay and demonstrating that there was no bioreactive TNFα in these samples (data not shown).

Changes in the acute phase response

The acute phase inflammatory response is an important TNFα-inducible element of the immune system and can be monitored by measuring CRP and IL-6 in serum samples. A single infusion of 5 mg/kg infliximab results

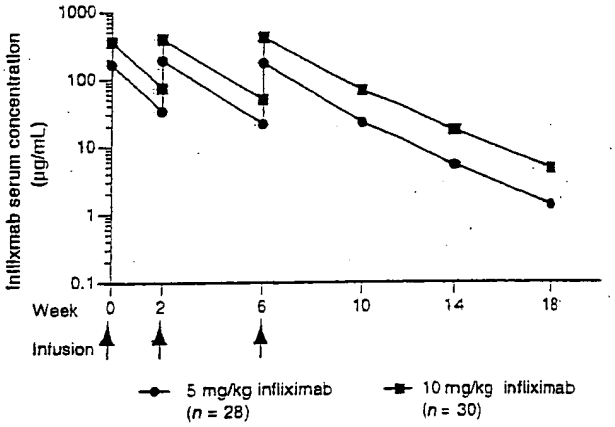

Figure 2. Profile of serum infliximab concentration vs. time for fistula patients who received three infusions of 5 or 10 mg/kg infliximab at weeks 0, 2 and 6 (arrows). Serum samples were obtained before and after each infusion and at the indicated time points after the third infusion. Each point represents the median result for that time period and dose group.

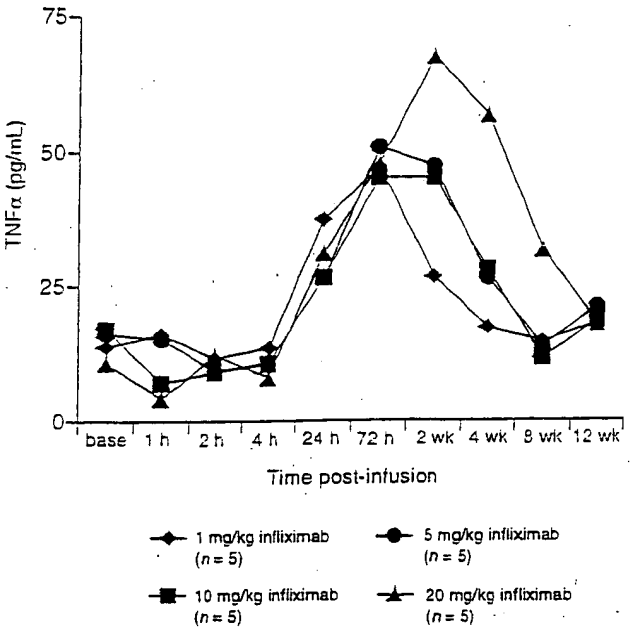

Figure 3. Concentrations of serum TNFα in serial samples collected from Crohn's disease patients in Study T11 following a single infusion of 1, 5, 10 or 20 mg/kg of infliximab. Each data point represents the mean for that time and dose group.

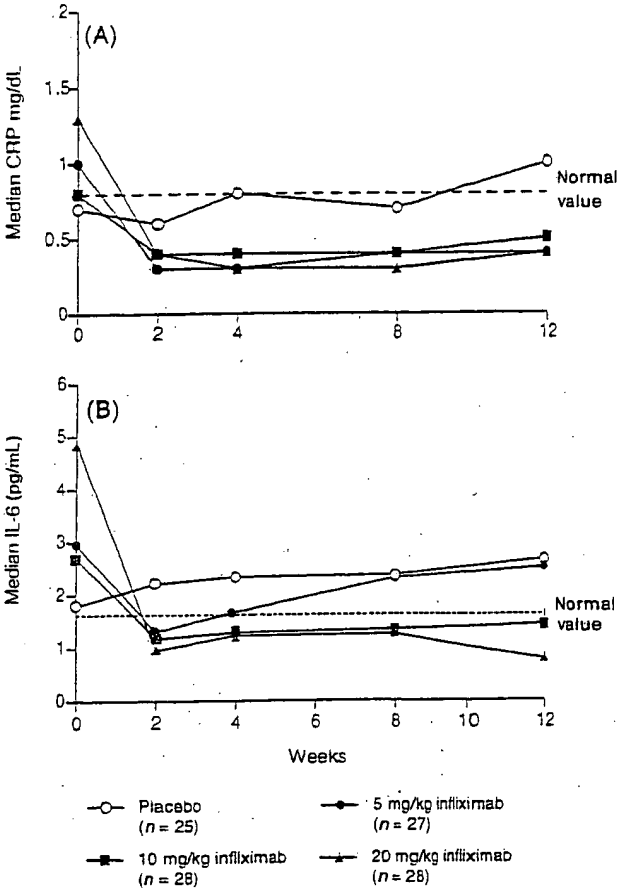

Figure 4. Median serum concentrations of CRP (Panel A) and IL-6 (Panel B) in Crohn's disease patients who received a single infusion of placebo, 5, 10 or 20 mg/kg infliximab. The median CRP (0.8 mg/dL) and IL-6 (1.6 pg/mL) concentrations in normal human serum are indicated by the dotted line on each graph.

in normalization of CRP levels by 2 weeks, which is maintained through 12 weeks (Figure 4a), while a gradual increase was observed for patients treated with placebo. IL-6 serum levels were also normalized at 2 weeks after infusion of 5 mg/kg, then gradually increased in the 5 mg/kg group, but remained within the normal range (1.6 pg/mL) in the higher dose groups (Figure 4b). No changes in IL-6 concentrations were observed in the placebo-treated group. Similar results were obtained in samples from T20 for both CRP and IL-6 serum levels (data not shown).

Differential and total WBC counts

The changes in differential cell counts for the 18-week period in study T20 are shown in Figure 5. Eosinophils and basophils comprised minor fractions of the total cell counts and did not show systematic changes over time. Baseline counts of monocytes, lymphocytes and neutrophils were 449, 1.452 and 6.424/mm^3, respectively (5 mg/kg dose group of T20). Increases in the median percentage of both monocytes and lymphocytes occurred in the infliximab treatment groups. In the 5 mg/kg infliximab group, the median lymphocyte percentage increased from 16.5% at baseline to 26.5% at 10 weeks (to 2.332/mm^3) while monocytes increased from 5.1% to 7.0% (to 616/mm^3) in the same time period. A corresponding decrease in the median percentage of neutrophils accompanied the increased lymphocyte fraction (from 6.424 to 5.430/mm^3). Both lymphocyte and neutrophil fractions returned toward the baseline profile at 14 and 18 weeks (8 and 12 weeks after the last infliximab infusion). Similar results were obtained from the T16 study (data not shown). The changes in differential cell counts were not due to altered total WBC counts (from 8.800/mm^3 at baseline to 8.100/mm^3 at week 18 for the 5 mg/kg dose group of T20; Figure 6). A modest decrease (T16) or no change (T20) was observed following single or multiple infliximab infusions, respectively.

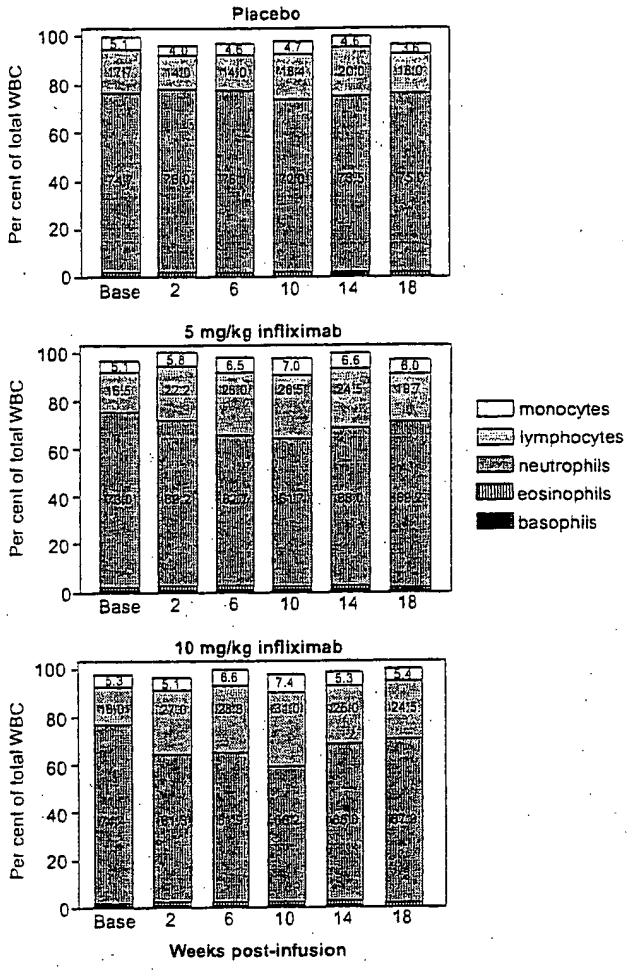

Figure 5. Differential WBC counts from Crohn's patients who received 3 infusions of placebo ($n = 31$), 5 mg/kg ($n = 31$) or 10 mg/kg ($n = 32$) of infliximab at 0, 2 and 6 weeks. The data for each cell type are presented as mean percent of total WBC.

Lymphocyte subsets

Peripheral blood cells were incubated with FITC or PE-conjugated antibodies specific for lymphocyte cell surface markers and analysed by FACS. As shown in Table 1, there were no marked trends observed in a comparison of the baseline and 6-week values in the infliximab-treated or placebo-treated patients, or by a comparison of the infliximab-treated and placebo groups. In addition, no changes in expression of activation markers (e.g. HLA-DR, CD57) were observed.

T cell function

T cells were studied from patients treated with single (T16) or multiple (T20) infliximab infusions. The results of these proliferation assays are summarized in Figure 7. Proliferation in response to specific antigens was not altered by infliximab treatment. Non-specific mitogens also showed no substantial changes from baseline, with the exception of ionomycin plus PMA. Response to ionomycin plus PMA increased proliferative responses approximately 2.3-fold in the infliximab group compared to a slight decrease in the placebo group.

Cytokine production by PBMC

IL-12 induces IFNγ, a marker of Th1 response and activator of macrophages. TNFα stimulates Th1 development and inhibits Th2 cell proliferation, while IL-10 blocks the activation of Th1 cells, and is considered a natural inhibitor of inflammation. Therefore, the

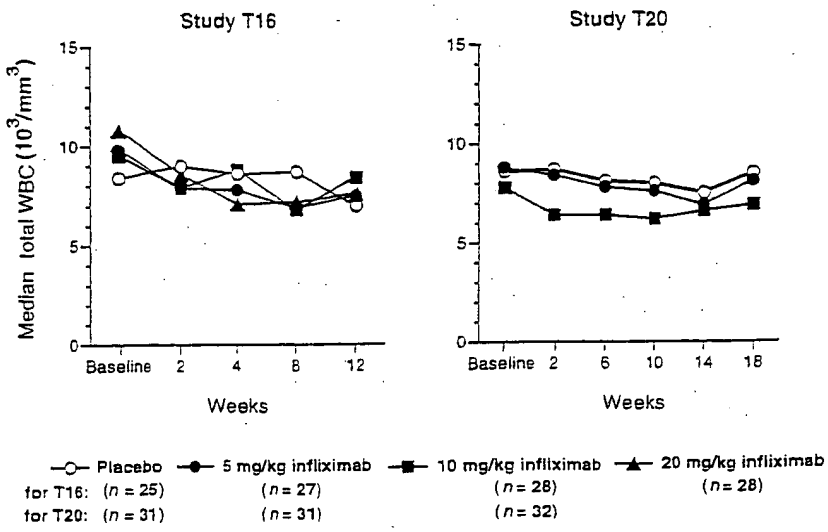

Figure 6. Total WBC counts for Crohn's disease patients who received placebo or infliximab infusions in Study T16 and Study T20. Each point represents the median value for that dose group and time point.

Table 1. Summary of lymphocyte subsets in Study T20

		Placebo		All infliximab-treated patients	
		0 week (n = 4) median %	6 weeks (n = 4) median %	0 week (n = 9–10) median %	6 weeks (n = 7) median %
T lymphocytes (%)					
(a)	CD3+	69.0	68.7	74.0	73.6
	CD5+	69.0	64.2	69.2	72.5
(b)	α/β +	65.7	60.2	69	67.3
	γ/δ+	0.8	8	4.5	3
(c)	CD4+	52.8	38.8	44.8	43
	CD8+	18.2	16.8	26.8	27.5
(d)	CD8+/CD28+	6.2	4.5	8	12.5
	CD3+/CD56+	1.5	3.8	4	5.5
	CD3+/CD57+	2.8	4.8	5.5	6
	CD3+/CD25+	7.8	8.5	6.5	4.5
(e)	Total HLA-DR+	22.2	25.0	22.0	28.7
	CD3+/HLA-DR+	6	8.5	9.5	10
	CD4+/HLA-DR+	4.5	5.5	5.5	6.5
	CD8+/HLA-DR+	1.2	3	3.2	3
B lymphocytes (%)					
	Total CD19+	14.8	16.2	8.8	13
	CD19+/CD5+	1	3	1.2	2
Non-T, Non-B lymphocytes (%)					
	Total	17.6	15.5	18.0	14.2
	CD56+/CD16+	9.5	8.8	10	10
	CD57+	2	4.2	3	2
	CD8	3.5	4.2	3	4.5

capacity of *in vitro* stimulated lymphocytes to produce IFNγ, TNFα and IL-10 provides information on the overall immune status of individuals. As shown in Figure 8, all three cytokines are increased in the stimulated PBMC compared to unstimulated control cell cultures at all time points. The *in vitro* production of IFNγ and TNFα by stimulated PBMC does not reveal any systematic trend after treatment with infliximab. This result suggests that the circulating population of lymphocytes capable of producing these cytokines is not affected by treatment with infliximab. With IL-10, there was a trend towards lower levels of production, particularly at later time points.

Cytokine production by LPMC

Biopsies were taken from inflamed areas, as well as from uninvolved (visually normal) areas of the colon. Isolated cells were incubated in the absence (control) or presence of anti-CD2 and anti-CD28 antibodies. The results, expressed as cytokine positive cells per 10 000 total cells, are shown in Figure 9. The number of cells from inflamed areas that could be induced to secrete Th1 cytokines (IFNγ and TNFα) decreases between 1 and 2 months and remained low through 4 months. Interestingly, the biopsies from uninflamed areas, although endoscopically normal, also had substantial numbers of cells that could be induced to produce IFNγ and TNFα and which also declined between 1 and 2 months after infliximab treatment. IL-10, which was not induced after stimulation of the LPMC, showed a trend toward lower number of cells producing this cytokine over the entire 4-month period of the study in both inflamed and uninflamed biopsies, although IL-10 producing cells in all biopsies were rare.

DISCUSSION

The pharmacokinetics of infliximab were found to be predictable and consistent over all three clinical trials in

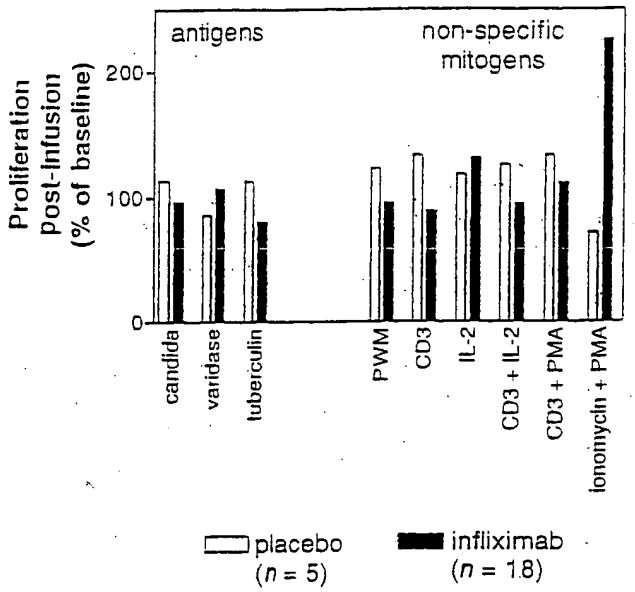

Figure 7. Effect of infliximab treatment on proliferative response to specific antigens and nonspecific mitogens. PBMC from placebo and infliximab-treated patients were isolated prior to treatment and 4 weeks after their last infusion and stimulated as indicated. Proliferation was determined by ^{3}H-thymidine incorporation. The median per cent proliferative response is shown relative to the response at baseline.

Crohn's disease. The terminal half-life of infliximab of 9.5 days is comparable with that of other IgG molecules.[9] Because of its long half-life and slow clearance rate (9.8 mL/h), infliximab remains detectable in serum for at least 8 weeks after a single infusion of 5 mg/kg. The prolonged bioavailability of infliximab, together with its high affinity ($K_a = 10^{10}\ M^{-1}$)[10] for TNFα results in effective and prolonged blockade of TNFα in patients. The median time to relapse in moderate to severe and fistulizing Crohn's disease patients is 2–3 months. Recently, Rutgeerts *et al.*[7] have shown that patients with moderate to severe Crohn's disease with an initial response to infliximab retain response and remission when re-treated every 8 weeks.

Measurements of free TNFα are hampered by the presence of TNFα:infliximab complexes in serum. However, this confirms the *in vivo* binding of TNFα by infliximab, and the circulation of these complexes for up to 12 weeks after a single infusion.[8] The experience in Crohn's disease patients provides clinical evidence that complexed TNFα is no longer bioactive. The immediate effect of TNFα neutralization by infliximab is evidenced by a rapid decrease of CRP and IL-6 levels. These inflammatory markers were normalized by 2 weeks

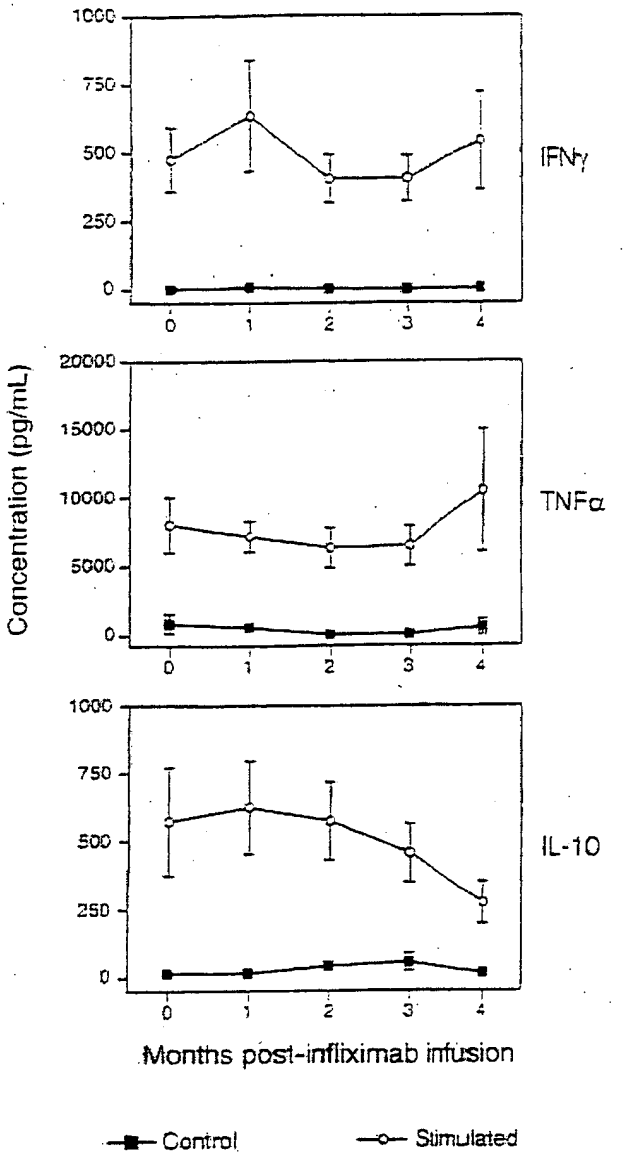

Figure 8. Cytokine production by PBMC isolated before and after treatment with infliximab. The concentration of IFNγ, TNFα and IL-10 were determined by immunoassay in the medium of PBMC cultures incubated with (stimulated) and without (control) concanavalin A and PMA. Each data point represents the mean ± S.E.M. (n = 13, 11, 13, 14 and 6 at 0, 1, 2, 3 and 4 months).

post-infusion, but other (unpublished) observations have demonstrated that these markers normalize within 48 h after administration of infliximab. Rapid onset of action is an important therapeutic advantage of infliximab compared with immunosuppressive therapies such as 6-mercaptopurine, for which the onset of response is often delayed and requires more than 4 months in 19% of patients.[11] Infliximab blocks IL-6, and thereby

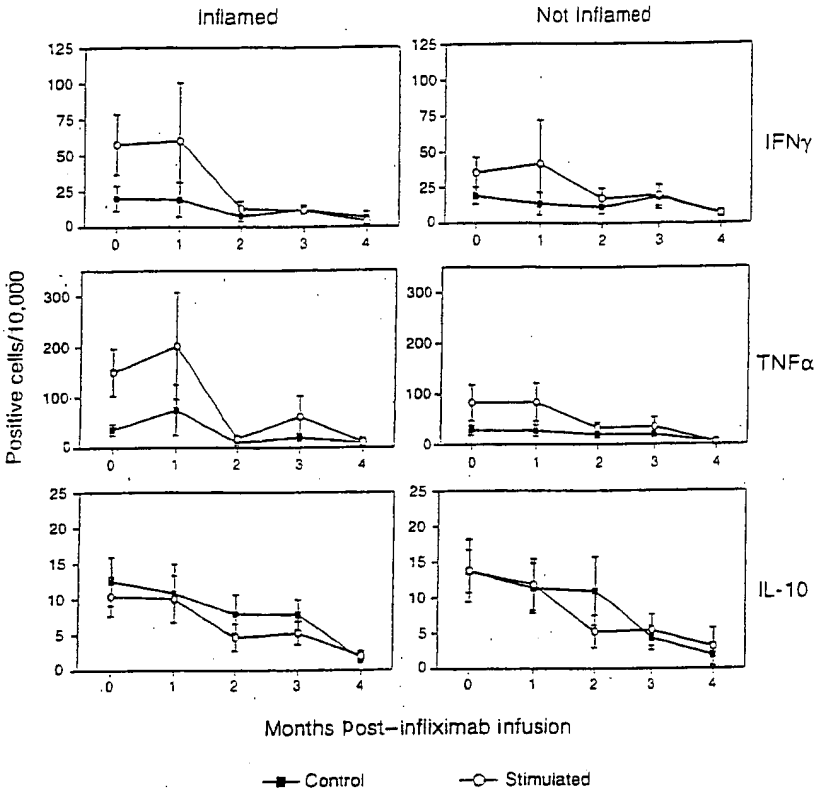

Figure 9. Effect of infliximab treatment on the number of cytokine-producing LPMC after stimulation. Prior to infusion and at the indicated times post-infusion, LPMC were isolated from biopsies representing inflamed and uninvolved areas of the colon and incubated in the absence (control) or presence of anti-CD2 and anti-CD28. The number of cells per 10 000 producing IFNγ, TNFα and IL-10 were determined by reli-spot assay, and each data point represents the mean ± S.E.M. (n = 19, 15, 11, 7 and 3 at 0, 1, 2, 3 and 4 months).

inhibits the production of the acute phase reactant CRP by the liver. When the signs and symptoms of disease return, usually after a 2–3-month period, both IL-6 and CRP increase. CRP concentrations remained below the normal range (0.8 mg/dL) in patients retreated every 8 weeks during a 36-week period, but not in placebo-retreated patients.[7]

The percentage of circulating lymphocytes and monocytes is increased between weeks 2 and 14 after infliximab infusions in T20, whereas the fraction of neutrophils is decreased. No significant changes were seen in the percentages of T cell subsets at 6 weeks after infliximab treatment. Recently, Maurice *et al.*[12] have described increased numbers of circulating CD4+ and CD8+ T cells in the blood of rheumatoid arthritis patients 3 days after treatment with infliximab. They suggest that homing of Th1 cells (secreting proinflammatory cytokines) into the inflamed synovium of rheumatoid arthritis patients is blocked by infliximab and that, as a result, these cells accumulate temporarily in the peripheral circulation.[12] Our data cannot confirm these results, as we focused on long-term rather than short-term effects.

Immunohistochemical studies have shown a significant reduction in the number of activated T cells in the lamina propria of the ileum and colon of Crohn's disease patients treated with infliximab.[3] The percentage of cells with positive stainings for ICAM-1 and LFA-1 were also significantly decreased.[3] Decreased numbers of inflammatory Th1 cells and TNFα positive monocytes and macrophages after infliximab therapy may be explained by increased *in situ* cell lysis, together with decreased cell influx due to down-regulation of adhesion molecules within the inflamed tissue. This study suggests that the homing of Th1 cytokine secreting T cells may also be inhibited by infliximab in Crohn's disease.

Our results demonstrated modest declines in the total WBC count following treatment with infliximab. However, the median WBC counts at all time points were within the normal range for adults (4500–11 000/mm^3). Mild leukopenia and more severe pancytopenia, while transient and reversible, have been reported with 6-MP[11] and azathioprine,[13] respectively. Similar changes have not been observed with infliximab. No prolonged changes have been observed in circulating lymphocyte subsets. The anti-inflammatory action of infliximab has local effects at the tissue level, resulting in architectural repair and tissue healing,[5] as well as systemic effects on the signs and symptoms of the disease, as shown by

decreases in CDAI and increases in IBDQ scores. The immune modulatory action of infliximab is mainly apparent at the tissue level, while the systemic immune function remains largely unchanged.

Proliferation assays of T cells performed 4 weeks after an infusion of infliximab demonstrate that these cells are immune-competent in response to antigenic and mitogenic challenges. The antigen-specific and mitogen responses indicate that infliximab treatment does not induce a generalized suppression of cellular immune functions, and that with certain stimuli, such as ionomycin plus PMA, enhanced proliferative responses can be seen. This is in agreement with the earlier findings of Cope *et al.*[14] which demonstrated improved proliferative responses of lymphocytes from rheumatoid arthritis patients after treatment with infliximab.

The *in vitro* production of the proinflammatory cytokines TNFα and IFNγ and the anti-inflammatory cytokine IL-10 by PBMC was studied after treatment with infliximab. Stimulation of PBMC with concanavalin A plus PMA resulted in increased INFγ and TNFα secretion, with little change observed over the 0–4 month post-infusion period. For IL-10, a trend towards lower production at later time points was evident. These results suggest that circulating Th1 lymphocytes are fully functional following infliximab treatment. We did not analyse the *in vitro* production of typical Th2 cytokines such as IL-4, IL-5 or IL-13 by PBMC after infliximab therapy. IL-10 is produced by both Th1 and Th2 cells and also by macrophages. The source of IL-10 measured in our *in vitro* assays is uncertain, and a possible effect of infliximab on Th2 cytokine production could therefore not be assessed.

In Crohn's disease patients responding to infliximab therapy, Plevy *et al.*[4] have shown that the numbers of LPMC-producing TNFα and IFNγ following CD2/CD28 activation are decreased over an 8-week period. In addition, this decrease in Th1 LPMC number was correlated with a decrease in CDAI (response) and endoscopic score. We confirm and further extend these findings. After a single infusion of infliximab the fraction of Th1 positive LPMC is decreased through 4 months after infusion in both inflamed and uninflamed areas. These results confirm that infliximab therapy may down-regulate proinflammatory cytokine responses in inflamed and uninflamed Crohn's mucosa.[4] Interestingly, Plevy *et al.*[4] have also shown that CD2/CD28-activated IFNγ and TNFα production from LPMC is down-regulated following infliximab therapy, while the production of IFNγ by PBMC is increased in clinically responsive patients. Therefore, and in agreement with findings in rheumatoid arthritis patients,[12] one effect of infliximab may be to prevent homing of Th1 cells from the periphery to the lamina propria.

In conclusion, the data presented from these clinical studies demonstrate that infliximab has profound local and systemic anti-inflammatory effects. The immune modulatory effects of infliximab are driven by the action of TNFα in the inflamed mucosa. Down-regulation of adhesion molecules within the lamina propria may inhibit homing of Th1 cells, resulting in an accumulation of lymphocytes in the circulation. Our results show no evidence of a generalized suppression of the immune system after infliximab therapy.

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Tumor necrosis factor in the pathogenesis of infectious diseases

BRUCE BEUTLER, MD; GEORGES E. GRAU, MD, PHD

Objectives: To review the immunologic role of the cytokines and the specific role that tumor necrosis factor (TNF) plays in response to infection. The influence of bacterial lipopolysaccharide on TNF, the cytokine cascade, and resultant pathologies are also reviewed.

Data Sources: A MEDLINE search of the international English language literature from 1960 to the present was reviewed, but data from the past 5 yrs primarily formed the basis for this review.

Study Selection: Those studies detailing the interaction of lipopolysaccharide, TNF, and other cytokines, and their roles in combating infection were emphasized. Investigations that described animal and human results served as the primary database.

Data Extraction: Animal studies were selected based on the relevance of the model to the pathogenesis of the human clinical syndrome. Where they provided supportive evidence, patient studies were selected on the basis of study design.

Data Synthesis: TNF plays a key role in the normal immune response to infection, limiting the spread of pathogens. Exaggerated physiologic responses occur under the influence of high concentrations of TNF that are released in response to overwhelming infection, resulting in aberrations in coagulation, cell adhesion, chemotaxis/transmigration, and vascular integrity. These pathologic effects may be inhibited by anti-TNF monoclonal antibodies and recombinant soluble receptor inhibitory proteins.

Conclusions: TNF exerts both physiologic and pathologic effects in response to infection; these events may lead to organ dysfunction and death. Anti-TNF therapies appear to attenuate the injurious effects of TNF. (Crit Care Med 1993; 21:S423–S435)

KEY WORDS: tumor necrosis factor; endotoxins; cytokines; antibodies, monoclonal; recombinant proteins; shock, septic; malaria; human immunodeficiency virus

The generation time of microbial pathogens is one thousandth to one millionth that of any mammalian species. For this reason, microbes enjoy an enormous advantage over their ponderous hosts. To the microbe and its descendants, the evolution of the host is so slow as to appear nonexistent: the host is quite incapable of emerging victorious in an "evolutionary footrace." To the extent that mammals survive infection, it is at the pleasure of the microbial invader, which may (in a teleologic sense) find it advantageous to permit the host to survive, rather than to kill it. So it is that pathogens often grow attenuated, and as is commonly the case, once-virulent parasites may even establish a symbiotic relationship with the host.

Although they cannot hope to outrace infectious organisms through evolutionary mechanisms, all complex metazoan organisms are shaped by the selective pressure of infection, and they have come to protect themselves through the development of anticipatory devices. This adaptation is best seen, perhaps, in the evolution of antibodies and T-cell receptors, which are capable of engaging virtually any foreign molecule that the host might encounter. Yet, even this elaborate system for rearrangement of genes, capable of generating an almost limitless variety of binding proteins, is easily defeated by the antigenic variation of trypanosomes, by the ability of many bacteria to seek refuge within cells, and by other mechanisms of escape.

The cytokines produced by the host may be viewed in light of the same principle: they were conjured into existence through selective pressures imposed by the microbial world, and have endured throughout the evolution of mammals because, on balance, they are of benefit in the containment of infection. Yet, the protection offered by cytokines may easily be defeated, and

From the Howard Hughes Medical Institute and the Department of Internal Medicine (Dr. Beutler), University of Texas Southwestern Medical Center, Dallas, TX, and the Department of Pathology (Dr. Grau), University of Geneva, Switzerland.

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Address requests for reprints to: Bruce Beutler, MD, Howard Hughes Medical Institute, 5323 Harry Hines Boulevard, Y5.210, Dallas, TX 75235-9050.

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may even be turned against the host, which is ever at the mercy of organisms that can quickly evolve the means to destroy it.

At times, the destructive effects of unfettered cytokine production seem accidental (e.g., when caused by the release of Gram-negative organisms from the colon as the result of appendicitis). On other occasions, cytokine release seems to further the invasive or infectious ambitions of a specific pathogen that evolved to kill or injure the host (e.g., in falciparum malaria, cerebral complications lead to paralysis, opisthotonos, and consequently, greater exposure to the mosquito vector and broader dissemination of the parasite). And on still other occasions, the release of cytokines may be concomitant with the attempt to deal with pathogens, and may ultimately reward the host with survival. The clinician is still challenged to determine which alternative applies in a given patient.

THE IDENTIFICATION OF TUMOR NECROSIS FACTOR AND IDENTIFICATION OF ITS ROLE IN INFECTIOUS DISEASES

Overwhelming infection frequently elicits a constellation of clinical problems, collectively termed the "septic syndrome." The septic syndrome is characterized by metabolic derangements (acidosis, hypoglycemia, hypertriglyceridemia), disseminated intravascular coagulation, hypotension (often leading to inadequate perfusion of vital organs), peripheral and pulmonary edema, and the death of cells in a variety of tissues. Both Gram-negative and Gram-positive organisms, as well as fungi, parasites, and certain viruses are capable of provoking the septic syndrome. To a large extent, septic syndrome caused by one organism is indistinguishable from that caused by another. Thus, the shock state that may occur in falciparum malaria is metabolically and histopathologically similar to that which occurs in *Escherichia coli* septicemia. This fact, in itself, indicates the existence of a "final common pathway" in septic syndrome. Indeed, much evidence now indicates that such a pathway does apply in septic syndrome, and its components have been partially deciphered.

Gram-negative organisms may cause septic syndrome when present at low strength, or when remote from the plasma compartment itself. They do so by virtue of their ability to produce "endotoxins" (lipopolysaccharides), which act as a powerful trigger for the development of septic syndrome. Administered in a pure form, lipopolysaccharide can reproduce the septic syndrome in experimental animals (1, 2).

While it was originally believed that lipopolysaccharide was directly toxic to cells and tissues of the host, it was eventually shown to be relatively innocuous, because cultured cells are not usually damaged by lipopolysaccharide, and a lipopolysaccharide-responsive hematopoietic system is required if lipopolysaccharide is to have a toxic effect. Mice of the C3H/HeJ strain, which are unresponsive to lipopolysaccharide as a result of a mutation localized to the fourth chromosome (3), are rendered responsive by adoptive transfer of hematopoietic stem cells obtained from endotoxin-sensitive animals. Conversely, endotoxin-sensitive C3H/HeN mice are rendered resistant to lipopolysaccharide if irradiated and transplanted with hematopoietic precursors obtained from C3H/HeJ mice (4). Therefore, a cell (or factor) of hematopoietic origin was believed to confer the toxic effect of lipopolysaccharide.

The role played by tumor necrosis factor (TNF) as a mediator of endotoxic shock was initially suspected because: (5) it is produced predominantly by hematopoietically derived cells (i.e., macrophages) (6–8); (9) TNF was produced in abundance in response to lipopolysaccharide challenge (10, 11); (12) the catabolic character of TNF (which was also isolated as "cachectin"), a mediator of lipopolysaccharide-induced suppression of lipoprotein lipase synthesis (10, 13); and (14) the observation that TNF was highly toxic to animals, provoking a shock syndrome similar to that initiated by lipopolysaccharide (15).

Proof of the involvement of TNF in endotoxic shock was adduced by studies in which animals were passively immunized against TNF and then injected with lipopolysaccharide. A three-fold shift in the dose-lethality curve was observed in these studies, suggesting that much of the toxic effect of lipopolysaccharide was derived from its ability to stimulate TNF production (16). The fact that passive immunization against TNF could not protect animals when extremely high doses of lipopolysaccharide were administered suggested that other toxic mediators might also be responsible for the septic syndrome, but that TNF was the first lethal principle involved, being produced in response to low doses of lipopolysaccharide. Subsequent studies, using other polyclonal (17) or monoclonal (18–22) antibodies that are capable of neutralizing TNF, as well as synthetic inhibitors of TNF action (23), confirmed that TNF was a central cause of endotoxic shock, and by inference, of the septic syndrome itself.

Very recent studies suggest that the shock-producing effect of TNF (as well as its protective effect against *Listeria monocytogenes*) is mediated through its 55-kilodalton receptor, in that homozygous deletion of this receptor causes an endotoxin-resistant state (H. Blüthmann, personal communication). On the other hand, mutant TNF molecules, which engage only the 55-kilodalton receptor but not the 75-kilodalton receptor, are far less toxic than the unmodified hormone (24).

In all likelihood, the toxicity of TNF depends on signals channeled through both of the receptors, which operate in a cooperative fashion.

Given that the septic syndrome in endotoxic shock is caused by TNF (which may act in conjunction with other toxic cytokines if the host is exposed to high concentrations of lipopolysaccharide), it is reasonable to ask whether the septic syndrome proceeds through elicitation of TNF in many other disease states as well. It appears that TNF is produced in response to many infectious organisms, both *in vitro* and *in vivo*. Once produced, TNF may exert a beneficial or deleterious effect, depending on the quantity in which it is produced and the time period over which its production is sustained. The acute release of large quantities of TNF, particularly when it is accompanied by other cytokines that potentiate its toxic effects, may lead to shock regardless of which organism originally triggered production. Therefore, passive immunization against TNF can be shown to mitigate the acute lethal outcome of malaria (resulting from cerebral involvement) in susceptible strains of mice (25) and *E. coli* sepsis in baboons (18).

On the other hand, the protective effect of TNF, produced in more modest quantities in response to other pathogens, is equally undeniable. Mice that are passively immunized against TNF are readily killed by infection with *Mycobacterium bovis* (26). Similar passive immunization studies demonstrated a protective effect of TNF in *Listeria monocytogenes* infection (27), in *Legionella pneumophila* infection (28), and in *Cryptococcus neoformans* infection (29) in mice. The ambivalent function of TNF is further illustrated by the observation that small doses of the protein (well below the quantities that are synthesized endogenously in septic animals or in animals with cerebral malaria) exert a protective effect against the lethal effect of a subsequent inoculum of *Plasmodium berghei* organisms (30). The protective effect of TNF in malaria has been described by Taverne et al. (12) and Clark et al. (14), who documented reduced parasite counts in the blood of animals injected with exogenous TNF (i.e., on the erythrocytic phase of the disease). Similarly, TNF infusion suppresses the increase in parasitemia in *P. berghei*-infected mice before it induces cerebral signs and acute death in cerebral malaria-resistant mice (see below). However, this beneficial effect of TNF is not at all restricted to the erythrocytic stages, but is extended to both the pre-erythrocytic and the sexual stages of the disease. TNF has potent inhibitory effects on the development of hepatic schizonts and on the infectivity of gametocytes. This dual role of TNF in malaria has to be kept in mind when intervention studies with TNF antagonists are considered (31, 32) (Fig. 1).

In assessing the apparent conflict between "good" and "bad" effects of TNF, it must be concluded that the strategy evolved for suppression and containment of infection carries with it an inherent risk. Although specific aspects of the host immune response have been associated with detrimental effects (e.g., a higher capacity to produce interferon γ is associated with the development of cerebral malaria, and a wider distribution of Vβ-8+ T cells yields susceptibility to shock induced by staphylococcal enterotoxin B), we are unable to comment as to why, in certain cases, such a liability was maintained (5, 9). While TNF may check the proliferation and spread of pathogens, it also may injure the host. To the extent that certain organisms provoke a lethal response, it may be considered that they have not achieved a state of symbiosis with the host.

THE PATHOLOGIC EFFECTS OF TUMOR NECROSIS FACTOR MAY BE SEEN AS LOGICAL CONCOMITANTS OF THE BENEFICIAL EFFECTS

Shock may be seen as an exaggeration of the responses that cytokines normally elicit in the course of host defense. By influencing coagulation, the margination, and transmigration of leukocytes, and by modulating metabolism, cytokines can assist the host in containment and eradication of infection. These processes may, of course, be carried to excess and work to the detriment of the host (Fig. 1).

Cytokines released by immunocompetent cells elicit complex responses in the vasculature of the host. Cytokines can be viewed as a means of communication between leukocytes and endothelial cells (33), insofar as they display discrete patterns of effects, which may be

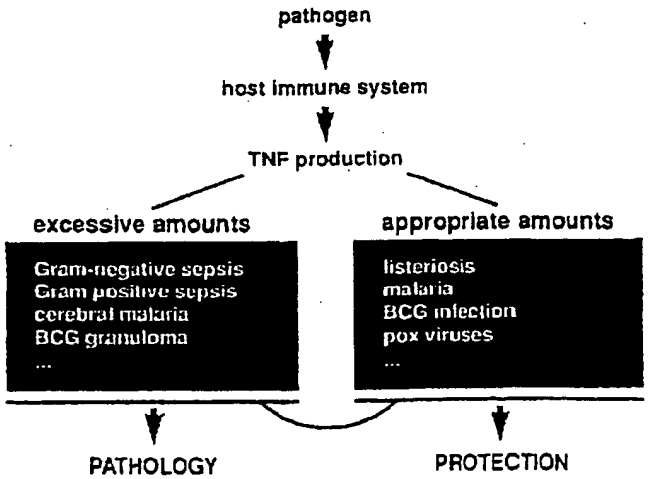

Figure 1. Dual roles of tumor necrosis factor (*TNF*) in infections. *BCG*, Bacille bilié de Calmette-Guérin.

proinflammatory, anti-inflammatory, or related to antigen presentation or to angiogenesis. Considerable overlap exists, in that most cytokines are able to evoke most of these vascular responses. Indeed, endothelial cells are able to react to most cytokines. They themselves are also capable of producing most cytokines, with the remarkable exception of TNF.

TNF exerts many effects on endothelial cells, direct as well as indirect, that are able to profoundly modify various systems. Important variables in TNF-induced endothelial cell alterations include:

a) *Coagulation.* TNF causes endothelial cells to release procoagulant activity (tissue factor), platelet-activating factor, and von Willebrand factor, which favor thrombosis. TNF downregulates the expression of thrombomodulin, which can block the assembly of protein C/protein S complexes, and thereby further decreases the anticoagulant property of endothelial cell surfaces. Fibrinolysis is also controlled by TNF, via modulation of the plasminogen activator-inhibitors and tissue-type plasminogen activator. It might be imagined that these procoagulant effects serve to isolate a nidus of infection from the circulatory system. Alternatively, the same effects occurring on a broader scale may foster the development of disseminated intravascular coagulation (34).

Other cytokines may influence these TNF-induced changes, as illustrated by the ability of interleukin-4 (IL-4) to inhibit the lipopolysaccharide-induced decrease in thrombomodulin (35). It has also been shown that administration of monoclonal antibodies against the CD3 antigen can result in thrombotic complications (36).

b) *Cell Adhesion.* The mobilization of leukocytes to combat infection demands that they bind to vascular surfaces in the relevant portion of the circulatory tree. In the presence of TNF, various sets of cell adhesion molecules (CAM) are either upregulated or expressed *de novo* on the surface of endothelial cells (37). Among the most important of these is ICAM-1. Besides mediating the adhesion of various leukocytes, ICAM-1 is one of the ligands for malaria-infected erythrocytes (38). Infected erythrocytes, together with mononuclear cells (39), become sequestered in brain capillaries that express high amounts of ICAM-1, induced by TNF that is released in severe malaria (40). Other blood elements, such as platelets, may also adhere via ICAM-1, impeding blood flow and fostering coagulation.

However, TNF does not simply cause upregulation of all cell adhesion molecules. In some cases, it triggers a change in conformation and/or affinity state of cell surface molecules. One of the ligands of ICAM-1 (LFA-1, a complex of CD11a and CD18) undergoes such a conformational change (41). In other cases, TNF activation results in reduced cell adhesion molecule expression. For instance, Mel 14 is lost from the surface of neutrophils through shedding when the cells are exposed to TNF. On loss of Mel 14, Mac-1 expression increases and CD18-dependent mechanisms become prevalent.

Some molecules are able to reduce TNF-induced inflammation. For example, GMP140 (or P-selectin/CD62), a molecule that is present in the A-granules of platelets, as well as in endothelial cells (in the Weibel-Palade bodies), has a peculiar and variable pattern of expression. GMP140 may be expressed as an integral membrane protein, and agents that induce translocation of GMP140 to the cell surface enhance neutrophil adhesion to endothelial cells. But three forms of GMP140 mRNA have been isolated, one of which encodes a secreted form of the protein. Secreted GMP140 significantly decreases neutrophil adhesion to endothelial cells or to plastic (42), and delays or prevents the release of superoxide anions from TNF-activated neutrophils. Most, if not all, adhesion molecules can be shed from cell membranes and can thereby modulate adhesiveness.

As with the cytokines that regulate their expression, there is a considerable degree of overlap in function among adhesion molecules. An example of this overlap is to be seen in the ability of endothelium leukocyte adhesion molecule-1 to promote T-cell adherence (43), even though this molecule was originally thought to be restricted to granulocytes and thus devoid of effects on T cells. Moreover, redundancy is illustrated by the fact that monoclonal antibodies directed against the cell adhesion molecules inhibit binding of the relevant leukocyte to TNF-activated endothelial cells, but never in a quantitative fashion. This inhibition is always substantial ($\lesssim$50% for anti-endothelium leukocyte adhesion molecule-1 monoclonal antibodies inhibiting neutrophil binding [44]), but it is by no means total. Furthermore, it has been clearly established that the addition of a mixture of antibodies to all known cell adhesion molecules does not interfere completely with leukocyte binding to endothelial cells. Therefore, unknown adhesion molecules remain to be characterized and are likely to play an important role in inflammatory processes.

c) *Chemotaxis / Transmigration.* Beyond attachment to the endothelium, leukocytes must leave the vascular compartment to effectively combat most pathogens. *In vitro*, TNF upregulates all the cell adhesion molecules and stimulates the release of all the chemoattractants that are relevant to transmigration. However, TNF can also affect leukocytes, for instance neutrophils, and act simultaneously as an activator of adhesion and as an inhibitor of migration by upregulation of CD11b on the neutrophil surface (45). *In vivo*, TNF causes margination of neutrophils, explaining the decrease in white

blood cell counts that is observed after TNF infusion, but no transmigration is induced (46). During inflammation, adhesion is accompanied by transendothelial cell migration. In some cases, however, there is an imbalance between these two phenomena and adhesion only is present, leading to intravascular sequestration, e.g., in cerebral malaria.

The precise mechanisms underlying this imbalance remain unclear, but it is likely that differential expression of cell adhesion molecules may play a role. Different sets of adhesion molecules seem to be required for mere adhesion vs. adhesion accompanied by transmigration. Vascular cell adhesion molecule-1 and GMP140 expression appear to act principally as mediators of adhesion, while ICAM-1 or endothelium leukocyte adhesion molecule-1 mediate both adhesion and transmigration (47). The loss of some adhesion molecules appears to be required for de-adhesion and ensuing transmigration, e.g., the loss of Mel-14 (48). In addition, the function of certain enzymes is critical to determine whether migration will or will not follow adhesion. Collagenase production, which is induced by TNF (49), can modulate migration by permitting transit through the basement membrane (50). Alternatively, migration can occur without known cell adhesion molecules. Under some conditions, endothelium leukocyte adhesion molecule-1 may not be required for migration across activated endothelial cells (51).

Inhibitors of the migration process have been described, just as inhibitors of adhesion are known to exist. The more recently characterized platelet-endothelial cell adhesion molecule-1 localizes to sites of cell-cell contact, promotes adhesion, and diminishes the rate of transendothelial migration (52).

On stimulation by TNF, endothelial cells release proteins, such as the colony-stimulating factors and interleukin-8 (IL-8). Besides their activity on hematopoietic cells, colony-stimulating factors can activate leukocytes and trigger their directed locomotion. Moreover, some of these molecules, especially G- and GM-colony stimulating factors, are able to bring about endothelial cell migration and neoangiogenesis. While promoting transmigration of neutrophils, IL-8 can also reduce neutrophil adhesion, thus acting as a component of a negative feedback loop (37). It has recently emerged that the leukocyte adhesion inhibitor is homologous to IL-8 (53). This leukocyte adhesion inhibitor/IL-8, along with transforming growth factor-β and GMP140, may be categorized with molecules that are able to mitigate the effects of TNF on neutrophil function.

d) *Other Effects on the Vasculature.* TNF triggers the release of prostaglandin E_2, prostacyclin I_2, thromboxane A_2, and nitric oxide (which exert vasodilatory effects) and endothelin (a powerful vasoconstrictor) from endothelial cells. These mediators can exert conflicting effects, and the net influence of TNF may depend on the location in which it is produced and the vascular bed with which it interacts. Beyond all effects on vascular tone, these terminal mediators of TNF action can affect platelet aggregation, which may either be enhanced or inhibited (Fig. 1). Finally, direct effects of TNF, such as cytotoxicity, and the ability to create a vascular leak (54–58), play important roles in acute infection.

The final effect of TNF on endothelial cells depends not only on the concentration of the cytokine, but also on the mode and duration of administration. For instance, the infusion of TNF via osmotic minipumps reproduced the whole range of activities that TNF can display. Depending on the dose and rate of administration, a proliferative response, the induction of perivascular hemorrhage, and finally, complete necrosis were each observed (59). These effects of TNF are, to varying degrees, each involved in endotoxicosis or cerebral malaria.

The involvement of TNF in the pathophysiology of sepsis has been extensively studied and recently reviewed (60). In a mouse model of cerebral malaria, it has similarly been shown that TNF plays a crucial role in the development of neurovascular lesions. Arguments also suggest a role for TNF in human cerebral malaria (31, 32, 61).

The role of nitric oxide has been evaluated, with respect to its influence on the course of cerebrovascular disease in malaria. Nitric oxide production is triggered by various cytokines, particularly TNF (62). However, production is diminished in the presence of lipopolysaccharide (63). The involvement of nitric oxide in the TNF-mediated pathogenesis of malaria is therefore complex. Nitric oxide can undoubtedly explain some of the early changes present in cerebral malaria (64), but probably not the end-stage lesions of the disease, which include brain hemorrhages due to microvascular endothelial cell lesions. This conclusion is based on the observation that N-monomethylarginine, an inhibitor of nitric oxide-synthase, has an aggravating, rather than protective, effect in mouse cerebral malaria (65, 66). The mouse model in which these studies were conducted is similar to the lethal cerebral malaria of humans, featuring multifocal encephalopathic changes and brain hemorrhages, a situation identical to that which occurs in human cerebral malaria (39).

Investigations of murine cerebral malaria prompted the suggestion that an effector mechanism involving platelets might account for the development of TNF-induced endothelial cell lesions. This mechanism consists of the adhesion of platelets to the surface of endothelial cells and, subsequently, the fusion of these platelets with the endothelial cells, resulting in

deposition of the cytoplasmic contents of the platelet within the cytoplasm of the endothelial cells. This process (67, 68) has been characterized *in vitro*, and its occurrence is predicated on two observations. First, after fusion has been induced *in vitro*, β-thromboglobulin, a platelet-specific protein, is observed within the cytoplasm of endothelial cells. Second, gpIIb-IIIa, a platelet glycoprotein, is transferred to the endothelial cell surface (Jinning Lou, PhD thesis, Beijing, China). The involvement of platelets in cerebral malaria pathogenesis accounts for the dramatic protective effect of anti-leukocyte function antigen-1 monoclonal antibodies, even when this monoclonal antibody is administered shortly before the time at which death would ordinarily occur. This monoclonal antibody protected malaria-infected mice, without reducing the number of mononuclear cells in brain vessels (69, 70). It thus appears that platelets are the critical effectors of TNF-induced neurovascular injury of cerebral malaria (71).

Viewed in this context, two arguments suggest that nitric oxide should have a protective rather than pathogenic role in cerebral malaria: a) nitric oxide can reduce platelet aggregation (72); and b) L-nitro monomethyl arginine treatment is known to increase platelet deposition on damaged endothelium *in vivo* (73).

INVASIVE STIMULI AND SIGNALING PATHWAYS THAT LEAD TO TUMOR NECROSIS FACTOR GENE EXPRESSION

Many different stimuli can elicit the synthesis and secretion of TNF protein. At least some of these stimuli utilize different signaling pathways and affect TNF production both by activating transcription of the TNF gene (and causing more rapid accumulation of newly synthesized TNF mRNA) and by enhancing the efficiency of TNF mRNA translation. In blocking the production of TNF through the administration of anti-inflammatory agents such as glucocorticoids (74, 75) or phosphodiesterase inhibitors (75, 76), clinicians actually disrupt signaling pathways, rather than causing any change in the TNF gene itself. An understanding of the mechanisms by which various toxins, drugs, and physical agents trigger biochemical cascades leading to the TNF gene would perhaps make more specific and effective therapy possible.

The TNF gene is but one of three members of a gene "family" derived from ancient tandem duplication events, and lying within the major histocompatibility complex (77). The lymphotoxin-α and lymphotoxin-β genes are closely linked to the TNF gene. The former gene encodes a protein with approximately 28% homology to TNF. Lymphotoxin-α is a product of T and B lymphocytes, and its production by other cells has not been reported. Lymphotoxin-β, a type-II membrane protein, is produced by these cells as well, and forms a heteromeric complex with lymphotoxin-α. The regulation of the lymphotoxin genes is less well-studied than the regulation of the TNF gene, as is the function of the proteins they encode. Below, we consider only the regulation of TNF.

The TNF gene is only accessible to the transcriptional apparatus in a limited subset of cell types. In most cells, the gene is highly methylated, and presumably condensed in a heterochromatic state. Accessibility of the TNF gene is developmentally controlled, and largely accounts for the tissue distribution of TNF production. Recent studies have shown that the TNF promoter is utilized in virtually all cell types, whether or not the endogenous TNF gene is accessible (78). When chloramphenicol acetyl transferase reporter constructs driven by the TNF promoter are transiently transfected into cells, chloramphenicol acetyl transferase expression results, indicating that the promoter is not active solely in macrophages, lymphocytes, or in other cells that are known to express the gene. However, only thymic T cells (79) and cells of the trophoblast (80) appear to express the TNF gene constitutively.

TNF gene transcription does not assure the production of TNF protein. Biosynthesis of TNF is restricted at the translational level, principally by sequences that reside within the 3'-untranslated region of the mRNA. Like many cytokine mRNA molecules (81), the TNF mRNA contains an extensive (uridine- and adenine-rich) element that strongly represses translation, acting through an as-yet-unknown mechanism (82). Cell activation, by lipopolysaccharide (83) or by ultraviolet light (84), can overcome this repression, allowing production of TNF to proceed.

While 2-aminopurine (85, 86) and certain phosphodiesterase inhibitors (75, 76, 87) repress transcription of the TNF gene, glucocorticoids exert a mixed action, preventing activation of both transcription and translation (74). This effect suggests that steroid hormones act at a very proximal level in the chain of events that follow cell activation.

What is the nature of the signaling pathways that are activated by microbial pathogens? The most studied pathway is that activated in macrophages by lipopolysaccharide. CD14, a glycerolphosphoinositol-anchored cell-surface protein, acts as a receptor for lipopolysaccharide, which is engaged either in a free form or as a complex with lipopolysaccharide-binding protein, a plasma protein of hepatic origin that serves to amplify the response to lipopolysaccharide (88). Thereafter, it is widely assumed that a second molecule, encoded by the lipopolysaccharide gene of mice, senses the presence of lipopolysaccharide and initiates a signal cascade. The

transcriptional response of the TNF gene is minimally dependent on modification of IκB (an inhibitory protein that complexes with nucleus factor-κB, and maintains this transcription factor in an inactive form within the cytoplasm). Once modified, IκB releases nucleus factor-κB, which translocates to the nucleus, and participates in transcription of the TNF gene (89). The nature of the translational activator/repressor system is unclear.

Other infectious agents utilize other signaling pathways to evoke the synthesis of TNF. *Borrelia burgdorferi* and *Treponema pallidum* each synthesize lipoproteins in which a single acyl chain is covalently attached to an amino-terminal cysteine residue (90–92). These lipoproteins (or synthetic lipopeptides of much smaller size) are able to stimulate TNF biosynthesis through a pathway distinct from that utilized by lipopolysaccharide. Thus, they are able to activate the TNF gene within macrophages derived from both C3H/HeN and C3H/HeJ mice. At high concentrations, the lipopeptides seem to activate signaling through the classical lipopolysaccharide pathway as well (93).

Still other bacterial products activate the TNF gene, through mechanisms that remain unexplored. Exotoxins derived from staphylococci (including enterotoxin A [SEA] and enterotoxin B [SEB]) (94, 95) and *E. coli* (Shiga-like toxin-I) (96) are capable of doing so, for example. The mortality rate that is associated with Gram-positive septic shock induced by injection of the superantigen, staphylococcal enterotoxin B, can be prevented by treatment with anti-TNF monoclonal antibody (5). However, Gram-positive septic shock takes place via somewhat different pathways; it involves the direct stimulation of T cells via superantigens. This contention is supported by four lines of evidence (9): first, T-cell derived cytokines such as IL-2 and interferon γ are detectable in the serum of SEB-challenged animals; second, cyclosporine A treatment effectively blocks the development of shock; third, T-cell-deficient severe combined immunodeficiency mice do not react with shock symptoms on injection of SEB; and fourth, this failure can be overcome by reconstitution of severe combined immunodeficiency mice with congenic immunocompetent T cells.

In the case of Shiga-like toxin-I, stimulation results in renal-specific expression of the TNF gene, which may partially explain the peculiar pathology of the hemolytic uremic syndrome (96).

Furthermore, soluble antigens released by malaria parasites appear to be major TNF inducers (97–100). These soluble antigens from rodent plasmodia are heat-stable molecules, distinguishable from lipopolysaccharide, that trigger TNF release *in vitro* and *in vivo* and that can kill mice made hypersensitive to TNF by D-galactosamine treatment. Medium from cultures of *Plasmodium falciparum* contains similar soluble antigens that induce TNF release from both human and mouse macrophages. Most importantly, these soluble antigens induce T-cell-independent antibodies that specifically block the ability of these antigens, but not that of endotoxin, to cause the secretion of TNF. These findings have two potentially important practical consequences. First, murine macrophages, whether derived from the peritoneal cavity or from a cell line, can be used to screen potentially toxic human malaria antigens. Second, the mouse can be used, not only to examine cross-reactions between toxic antigens and to study antigenic variation, but also to screen antigens in vaccination experiments as potential candidates for disease-preventing vaccines in man. These data led Playfair and colleagues (101) to the concept of antidisease vaccine.

Viruses are capable of activating the TNF gene as well. Influenza virus (102, 103), Sendai virus (104), Theiler's encephalomyelitis virus (105), and human immunodeficiency virus (106, 107) have each been reported to induce TNF biosynthesis. It is possible that TNF synthesis, witnessed in the context of viral infection, may actually suit the purposes of the virus (which, as a credit to its short generation time, may "outwit" protective mechanisms of the host that ordinarily prevent TNF synthesis). On the other hand, clear evidence has been brought forth indicating that certain viruses (notably the Shope fibroma virus) actually synthesize a strong bivalent inhibitor of TNF, having captured the TNF receptor gene from the mammalian genome and utilized it as a secreted TNF-binding protein (108). Mutations of this viral "TNF inhibitor gene" markedly diminish pathogenicity of the virus. This finding is consistent with separate observations that TNF can effectively counter viral replication or cytopathic effect (109–111), and strongly suggests that the virus has evolved an effective strategy to counter the host response to its presence.

TUMOR NECROSIS FACTOR IN HUMAN IMMUNODEFICIENCY VIRUS INFECTION

Much debate has centered on the role played by TNF in retroviral infections, and in particular, on the question of whether HIV is capable of inducing TNF gene expression within macrophages or other target cell populations. Circulating TNF has been measured in the plasma of HIV-infected individuals (112–116). However, it remains to be seen whether this TNF results from direct induction by the retrovirus or from intercurrent infectious processes. The *in vitro* infection of monocytes or lymphocytes with HIV has variously

been reported to induce TNF synthesis (106, 107, 117–119), or to induce no TNF synthesis (120, 121). The design of more sensitive assays for TNF may help to clarify whether HIV is actually an inducer, yet will leave open the question of whether TNF production is biologically significant.

TNF production in HIV-infected individuals is said to terminate the latent phase of infection, permitting rapid multiplication of the virus and concomitant destruction of the CD4 cell population (106, 122). This process is believed by some to involve the generation of free radicals within cells targeted by TNF, subsequent activation of NF-κB, and ultimately, activation of transcription from the retroviral long terminal repeats. It has been proposed that free-radical scavengers such as N-acetylcysteine, which increase cytoplasmic glutathione concentrations, might prevent activation from taking place through this sequence of events (123). Alternatively, the use of reagents that interfere with TNF production or activity has been proposed as a means of arresting the progression of HIV infection. Clinical trials have been initiated using each approach.

TRANSDUCTION OF THE TUMOR NECROSIS FACTOR SIGNAL: THE TUMOR NECROSIS FACTOR RECEPTOR "FAMILY"

Once secreted, TNF is capable of engaging two types of receptor. The larger of these receptors is 75 kDa in size, and the smaller is 55 kDa in size, the difference being largely attributable to differences in glycosylation. The trimeric TNF molecule acts to cross-link receptor-monomers on the cell surface, and it is receptor aggregation, rather than an allosteric change elicited in the monomers, that triggers a biological response.

Both of the TNF receptors display a four-fold cysteine-rich repeat in the extracellular domain, and so may be identified as members of a larger family of receptor proteins, which includes the Fas antigen, CD40, the nerve growth factor receptor, and several other proteins of unknown function (108, 124, 125). The Fas antigen and CD40 have clear immunologic functions. The former appears to function in the elimination of T cells in the thymus, and mutations of Fas lead to a lymphoproliferation syndrome in mice (126), in which T cells of an unusual type accumulate to very high concentrations in peripheral lymphoid organs. The latter phenomenon is expressed on B cells, and is required for the normal switch from immunoglobulin (Ig) M production to the production of IgG, IgD, and IgA. Mutations of the CD40 ligand (an integral membrane protein on T cells) lead to an unusual X-linked immunodeficiency in humans.

The critical functions of the TNF receptors in immunity remain unknown. The cytoplasmic domains of the two TNF receptors are entirely dissimilar, and therefore, are presumed to transduce different signals. The 75-kilodalton receptor seems to stimulate T-cell proliferation *in vitro*, whereas the 55-kilodalton receptor transduces the well-known cytolytic effect of TNF. The latter receptor is also clearly involved in mediating at least some of the toxic effects of TNF, as well as protection against *L. monocytogenes* infection, since mutational deletion of this receptor leads to diminished sensitivity to lipopolysaccharide, and to greatly enhanced sensitivity to *Listeria* organisms (H. Blüthmann, personal communication).

INHIBITION OF TUMOR NECROSIS FACTOR ACTIVITY BY MONOCLONAL ANTIBODIES

Clinicians are now in a position to administer TNF, or to prevent the synthesis or block the activity of TNF. The administration of TNF in the setting of infection may yet be deemed useful, but as of the time of this writing, no clear indications for TNF administration have been set forth. On the other hand, there are clearly circumstances in which the inhibition of TNF synthesis or activity seems desirable, if one accepts that any beneficial effect of the protein in combating disease may be outweighed in the short run by its propensity to cause shock, tissue injury, and death. Inhibition of TNF activity may be achieved with great specificity.

Monoclonal antibodies against TNF are currently being tested in the treatment of septic shock. Both murine monoclonal antibodies and "humanized" monoclonal antibodies are under study. Early indications are that these reagents offer significant protection against a lethal outcome in humans. Obviously, they might be of use in many other infectious and inflammatory settings.

The efficacy of anti-TNF antibodies may rest, in part, on their ability to interrupt the cycle of TNF production that results from macrophage stimulation via an autocrine route. In mice infected with *P. berghei*, it has been demonstrated that anti-TNF not only abolishes the biological activity of TNF in the circulation (and hence, the associated pathology), but also prevents TNF mRNA accumulation in the brain and lymphoid organs. A similar finding is evident in mice infected with Calmette-Guérin bacillus (26).

Neutralizing monoclonal antibodies against TNF evidently affects blockade of TNF activity by steric inhibition of receptor binding, or by distortion of the trimer, such that the receptor no longer recognizes the binding sites. Surprisingly, it does not hasten the clearance of TNF from the circulation. A clinical trial of the

safety of anti-TNF monoclonal antibodies in African children with *Plasmodium falciparum* infection (127) demonstrated that circulating TNF concentrations (measured by immunoassay) actually increased after treatment with anti-TNF monoclonal antibodies, although the clinical effects of TNF were inhibited, as indicated by a significant reduction of fever. While free TNF is rapidly cleared from the circulation (128) as a result of its association with plasma membrane receptors, TNF that is maintained as a complex with anti-TNF monoclonal antibodies may circulate for a prolonged period of time and is ultimately disposed of in a different manner (Fig. 2).

While very effective in blocking the activity of TNF, monoclonal antibodies might conceivably pose problems related to their antigenicity (particularly in the event that murine reagents are employed) and might also prove injurious because they form circulating immune complexes with the target protein. These considerations may be of minimal importance given the immediacy of the threat posed by excessive TNF production in sepsis or cerebral malaria. However, they may be more cogent under circumstances in which long-term inhibition of TNF activity is desired.

INHIBITION OF TUMOR NECROSIS FACTOR ACTIVITY BY RECOMBINANT INHIBITORY PROTEINS

The cloning of the TNF receptors allowed the design of highly specific, nonantigenic ligands for TNF that prevent its interaction with the plasma membrane receptors through a competitive mechanism. By joining DNA sequences coding for the TNF receptor extracellular domain to DNA sequences coding for a portion of the mouse (129) or human IgG heavy chains, synthetic genes coding for bivalent TNF-binding proteins were produced. These artificial TNF ligands, when expressed, were found to bind TNF with exceedingly high affinity, reflecting the fact that they combine with two separate receptor binding sites per trimer. The recombinant proteins circulate with a half-life that varies depending on the cellular source and purification technique, but that exceed 48 hrs (129). Inhibitors can be administered to mice from an external source, produced endogenously as the product of a transgene (130), or produced endogenously as the product of a gene introduced through the use of an adenoviral vector (J. Kolls and B. Beutler, manuscript in preparation). They gain access to the systemic circulation when administered by any parenteral route, and also cross the placenta (80).

TNF inhibitors of this sort offer a potential approach to the therapy of diseases in which TNF is produced over a long period of time. They have the advantage of being nearly or entirely nonantigenic. Of theoretical concern, however, is the possibility that autoantibodies against the TNF receptor, some of which may have agonist potential, might be generated in response to the administration of recombinant material. Moreover, it is possible that much of the TNF that becomes bound to such molecules is not cleared, but is merely stored in a circulating form and later released.

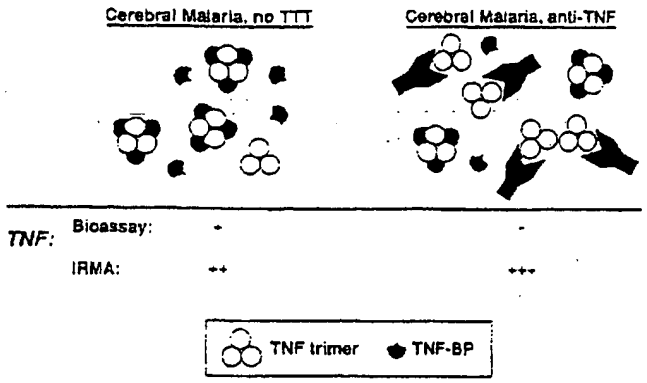

Figure 2. Biological vs. immunological assays for tumor necrosis factor (*TNF*): mechanism of action of anti-TNF monoclonal antibodies *in vivo*? *IRMA*, immunoradiometric assay.

CONCLUSION

The effects of TNF in infectious diseases have recently come into much sharper focus. Both the beneficial effects of TNF and the harmful effects are better understood than they were a decade ago, when this protein was first isolated as a pure molecular species. Perhaps it is not surprising to learn that the "good" and "bad" effects of TNF are really one and the same, that a good medicine can be harmful if applied at the wrong time, or at an improper dose. All the while, our understanding of the molecular events that control TNF synthesis have grown, together with our understanding of the molecular events that TNF may trigger. Details of the tertiary and quaternary structure of TNF and its receptors have enabled molecular biologists to fashion novel inhibitors of the protein. It is to be expected that all of these advances will have a major impact on the treatment of human diseases to which TNF, or the lack of TNF, must contribute.

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Short communication

Tumor necrosis factor as a mediator of inflammation in influenza A viral pneumonia

Randall L. Peper[1] and Hana Van Campen[2]

[1]*University of Illinois at Urbana-Champaign, College of Veterinary Medicine, 2001 South Lincoln Avenue, Urbana, IL 61801, and* [2]*Department of Veterinary Sciences, University of Wyoming, Laramie, WY 82070, U.S.A.*

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Peper, R. L. (University of Illinois at Urbana-Champaign, College of Veterinary Medicine, Urbana, IL 61801, U.S.A.) and H. Van Campen. Tumor necrosis factor as a mediator of inflammation in influenza A viral pneumonia. *Microbial Pathogenesis* 1995; **19**: 175–183.

The role of tumor necrosis factor α (TNFα) in the pathogenesis of influenza A viral pneumonia was examined. CD-1 male mice were challenged intranasally with influenza A virus A/PR/8/34 (H1N1) and administered rabbit anti-mouse TNFα-specific-neutralizing antibodies intraperitoneally. The effect of treatment on virus titer, TNFα levels, morbidity, mortality, and on pathologic lung lesions were compared with sham-treated controls. The severity of gross and histologic lung lesions positively correlated with the peak bronchoalveolar TNFα levels and was ameliorated with anti-TNFα treatment. Survivorship was prolonged in mice given a lethal dose of virus by treatment with TNF-α neutralizing antibodies. Reduction of TNFα levels by treatment with TNFα-antibodies did not affect virus titers in the lung. These results suggest that TNFα is a mediator of pulmonary inflammation during influenza A viral pneumonia, but may not play a significant anti-viral role in influenza pneumonia.

Key words: TNFα; influenza; pneumonia; inflammation; treatment; mice.

Introduction

Influenza pneumonia is the most common complication of influenza A viral infections in humans. An estimated 10 000 to 20 000 deaths occur each year in the United States from influenza and resulting pneumonia.[1] Although primarily an infection of the upper respiratory tract, influenza A viruses can infect cells present in the lung resulting in a primary viral pneumonia. Affected areas of the lung are characterized by an interstitial pneumonitis with a leukocytic cell infiltrate in the submucosa of airways and in alveolar septae. These changes impair oxygen exchange and patients may rapidly succumb to hypoxemia. The inflammation of influenza viral pneumonia coincides with the appearance of viral antigens in alveolar macrophages as well as

Author to whom correspondence should be addressed: Dr. Hana Van Campen, Department of Veterinary Sciences, University of Wyoming, 1174 Snowy Range Road, Laramie, WY 82070, U.S.A.

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respiratory epithelial cells and pneumocytes. The presence of virus in macrophages suggest an important role for these cells in the pathogenesis of this disease. Macrophages release soluble factors or cytokines which mediate the host's response to infection. These cytokines signal other leukocytes resulting in the influx of these cells into infected tissues. The development of the interstitial cellular infiltrate in viral pneumonia may reflect the release of cytokines from influenza virus-infected macrophages.

TNFα is a cytokine produced and secreted principally by cells of the macrophage/monocyte lineage. This factor has multiple biological activities with potentially beneficial and harmful effects on the host during viral respiratory infection.[2] Investigators have shown that TNFα has antiviral activity against a wide range of RNA and DNA viruses.[3] TNFα inhibits viral replication by at least two mechanisms: (1) direct lysis of virus-infected cells,[4] and (2) the induction of an anti-viral state in uninfected cells.[5–7] These activities may benefit the host by decreasing viral replication in the lung.

TNFα mediates a wide spectrum of inflammatory and immunologic functions. *In vitro* studies have shown that TNFα attracts, activates, and enhances the cytotoxic actions of neutrophils, macrophages, cytotoxic T lymphocytes and NK cells.[8–14] TNFα induces the release of or synergizes with other immune regulators such as IL-1, IL-2, IL-6 and IL-8 stimulating a multitude of secondary actions.[10,11,15] By regulating inflammation, TNFα may control virus-induced injury during infection and tissue repair as the infection resolves. If present in high levels, TNFα may contribute to overwhelming inflammation, promote extensive tissue damage and lung consolidation in influenza pneumonia.

In vitro TNF-specific bioassays performed by Nain *et al.* indicate that influenza virus stimulates TNFα production by alveolar macrophages.[16] TNFα has been detected by Vacheron *et al.* in bronchoalveolar washings from virus infected mice.[8] These studies indicate that this cytokine is released in response to influenza A virus. In this study, we tested the hypotheses that: (1) TNFα is released in the lungs in response to influenza A virus infection; (2) TNFα mediates pulmonary inflammation and, thus, the lesions of influenza pneumonia; and (3) TNFα has antiviral effects against influenza A virus within the lungs.

Results

TNFα levels within the lung

To determine the levels of TNFα in the lungs of influenza virus-infected mice, lavage samples and cell-free homogenates were assayed for TNFα by ELISA. TNFα levels in lung lavage peaked 3 days post-infection (p.i.) (247.5 ± 74.6 pg/ml) (mean ± SEM) dropping to pre-infection level by 5 days p.i. (Fig. 1A). The highest mean concentration of TNFα detected in lavage fluid and the standard errors of the means were comparable to those reported by Vacheron and Hennet.[8,17]

Assessing TNFα levels in broncholavage fluid was difficult due to wide variation between measurements at a given time point. Variability between samples was reduced when TNFα was assayed in lung tissue homogenates and compared per gram of tissue. Since TNFα levels in the tissue homogenate represented cytokine concentration in the entire lung (parenchyma and airways), not just the airway compartment, this method was used for the remainder of the study.

Cell-free lung homogenates, from mice 4 days p.i., were analyzed for TNFα (Fig. 2). TNFα levels in the lungs of virus-infected mice (2295 ± 145 pg/g) ($n=5$) were significantly elevated over those of control uninfected mice (423 ± 54 pg/g) ($n=5$;

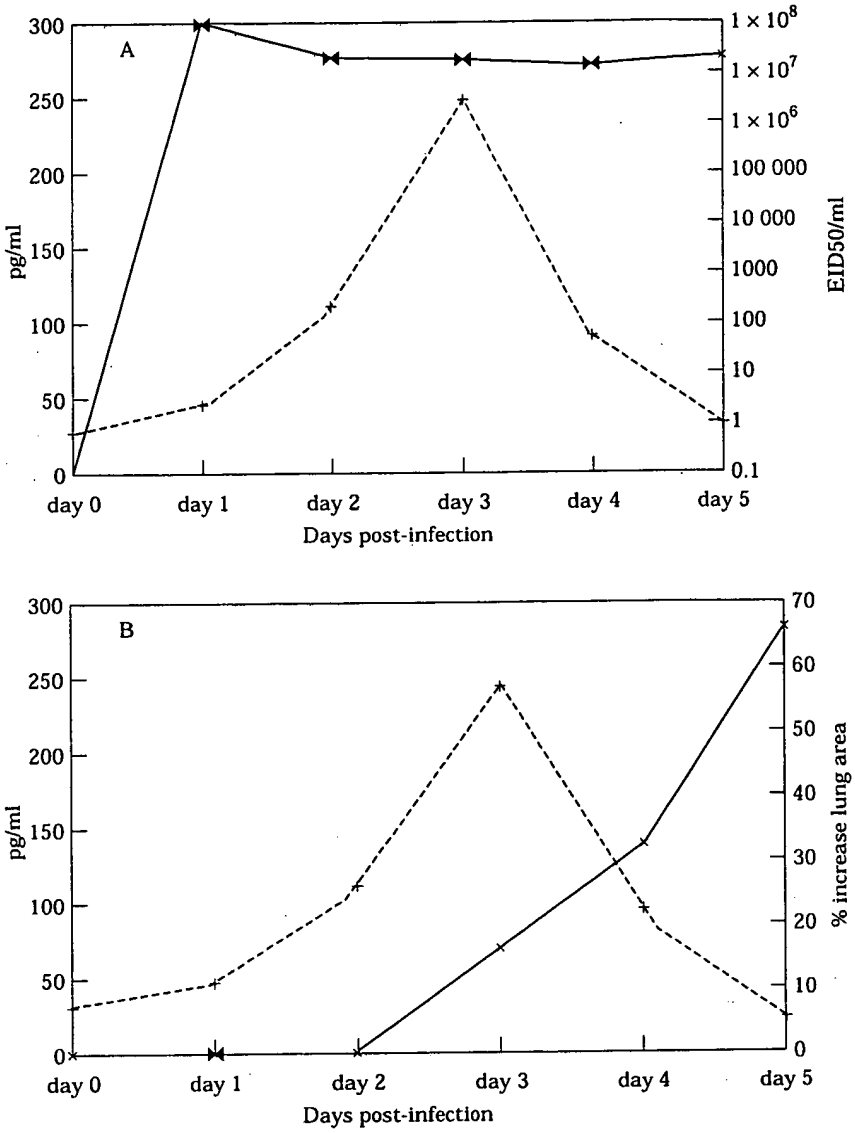

Fig. 1. Kinetics of TNFα levels (+—+, pg/ml) in lung lavage of influenza A virus-infected mice, days 0–5 p.i. (A) Compared with virus titer (×–×, EID50/ml) in homogenized lung tissue. (B) Compared with mean cross-sectional area of the lung (×–×, %) ($n=5$ for each time point; total = 30 mice).

$P=0.0003$). Treatment with a single dose of rabbit anti-mouse TNFα-neutralizing antiserum at the time of virus inoculation, decreased levels of TNFα in lung homogenates (1037 ± 145 pg/ml), compared to infected, sham-treated mice ($P=0.0008$) (Fig. 2). These results confirm that TNFα levels are increased in broncholavage fluid and cell-free lung homogenate during a productive influenza virus infection. Our findings indicate that the production of TNFα within the lung is modulated by TNFα neutralizing antiserum treatment.

TNFα antiserum treatment and survivorship

Treatment with a single dose of TNFα neutralizing antiserum, at the time of virus inoculation, delayed the onset of clinical signs and prolonged survivorship by 24 h

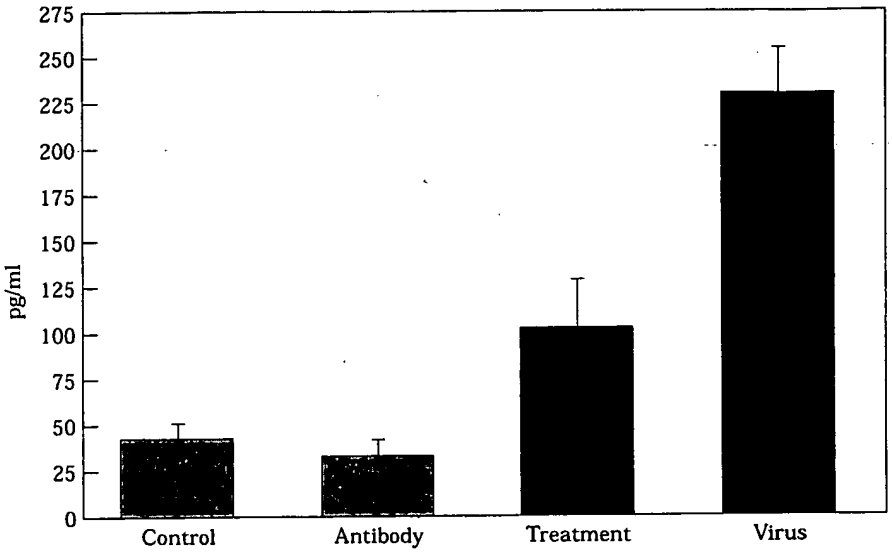

Fig. 2. The levels of TNFα as measured by ELISA, in cell-free lung homogenate day 4 p.i. Treatment with rabbit anti-murine TNFα neutralizing antibodies significantly ($P < 0.001$) reduced TNFα levels, as compared with cell-free lung homogenate from virus-inoculated, sham-treated control mice ($n = 5$).

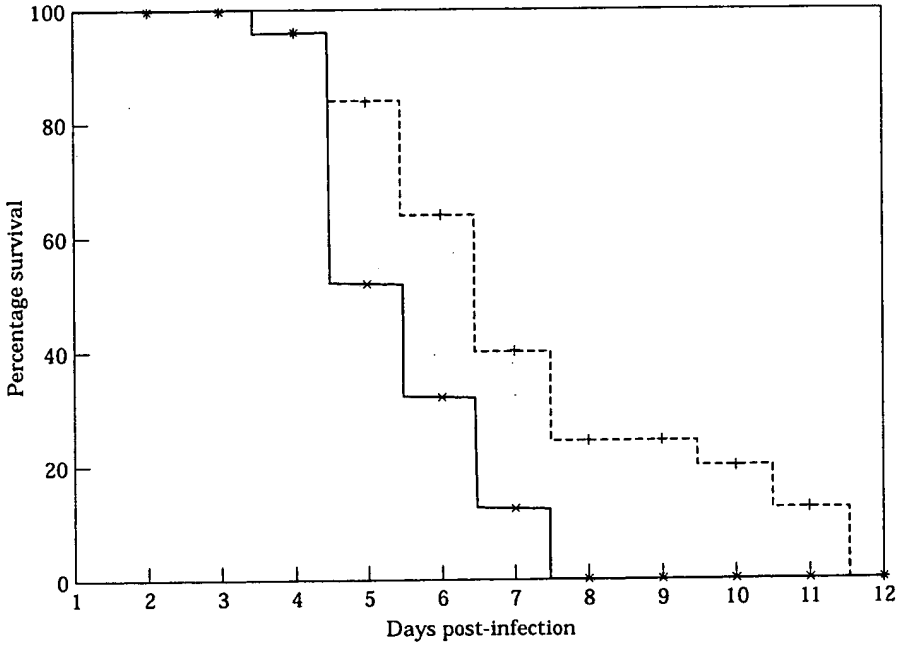

Fig. 3. Survival curve of virus-infected mice days 0–12 p.i. All sham-treated, virus-infected mice (×–×) died between days 4 and 8 p.i. Survival was extended for antibody treated mice (+–+) by approximately 24 h ($n = 15$ mice).

p.i. (Fig. 3). Although all mice treated with TNFα antiserum died ($n = 15$), 20% were surviving at day 10 p.i. In contrast, all sham-treated, virus-infected mice became moribund by day 8 p.i. ($n = 15$).

TNFα and lesion development

The viral infection resulted in the development of pneumonia within 5 days p.i., as evidenced by a progressive increase in gross and histologic lung lesions and in lung

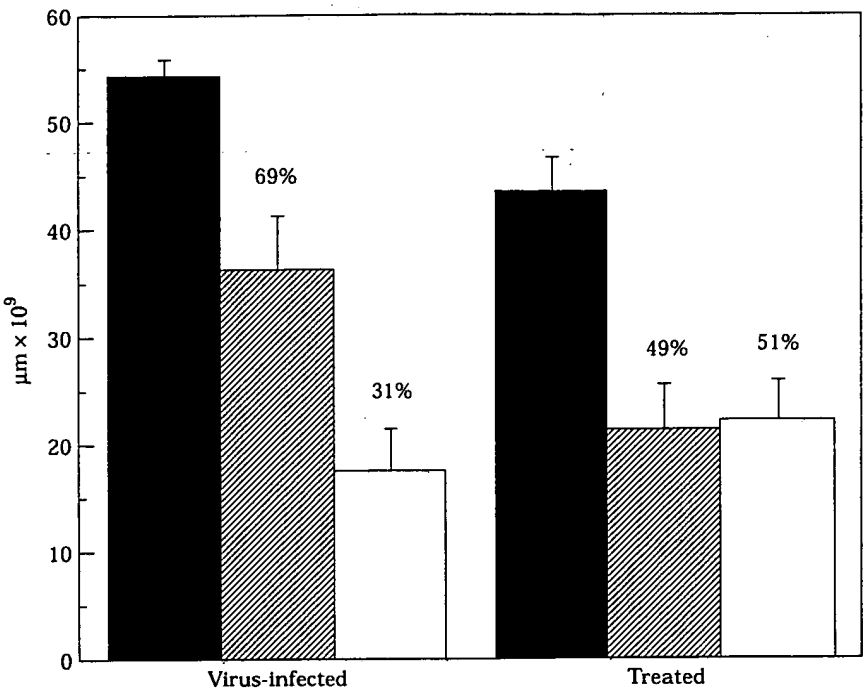

Fig. 4. Lung lesion severity at day 5 p.i. and antiserum treatment. Treatment resulted in both relative and absolute decreases in total cross-sectional surface area of the left lung lobe, as measured by morphometric analysis. Antibody-treated, virus-infected mice (treated) ($n = 5$/group) had significant decrease in areas of hypercellular infiltrate and comparable increases in normal appearing lung tissue. (■) total; (▨) hypercellular; (□) normal.

cross-sectional surface area ($n = 5$). The kinetics of TNFα expression in the lung were compared to the development of lung inflammation. Overall, inflammation (percent increase in total lung area) and gross lesion development paralleled TNFα levels in broncholavage fluid TNFα (Fig. 1B). TNFα levels rose by day 1 and peaked on day 3 p.i., while both gross lesions and lung area increased from day 3 through day 5 p.i. These results are similar to those of Hennet *et al.*[17] and suggest a correlation between TNFα and pulmonary inflammation.

TNFα antiserum treatment and lesion severity

Reduction of TNFα levels in the lung with TNFα-neutralizing antibodies was associated with a decrease in severity and extent of gross and histologic lesions on day 5 p.i. Treatment with TNFα-neutralizing antibodies decreased the mean lung cross-sectional surface area, as well as the relative and absolute areas of hypercellular infiltrate. Corresponding increases in normal appearing tissue were found in antiserum-treated, infected mice compared to sham-treated, infected controls (Fig. 4). These results reflected a decrease in overall lung inflammation with decreased levels of TNFα.

TNFα modulation of virus titer

Virus titers rose rapidly in the lungs peaking on day 2 p.i. Titers remained high through day 5 of the experiment indicating an active infection (Fig. 1A). Alteration of TNFα levels within the lung by treatment with TNFα-neutralizing antiserum did not appear to influence virus titers (Fig. 1A). Virus titers in the lung homogenates of sham-treated, infected mice ($1.2 \times 10^6 \pm 3.8 \times 10^5$ EID50/g) (mean ± SEM) on day 4

p.i. were not significantly different from those of treated mice ($9.9 \times 10^5 \pm 4.2 \times 10^5$ EID50/g) suggesting that TNFα may not influence virus replication *in vivo*.

Discussion and conclusions

Evidence suggests that TNFα is produced in the lungs during the course of influenza infection. Most studies to date have focused on the *in vitro* relationship between influenza virus and TNFα release. Macrophages are stimulated to release TNFα in response to live virus[18] or purified virus components[19] *in vitro*. Additionally, influenza virus stimulates the accumulation of cytoplasmic TNFα mRNA in macrophages.[16] These *in vitro* studies suggest an important role for TNFα in viral pneumonia; however, *in vivo* evidence is limited.

Two recent studies reported elevation of TNFα in broncholavage fluid from influenza virus infected mice.[8,17] These studies suggest that TNFα levels increase in the airways of the lung following influenza virus infection. Our study has confirmed the rise of TNFα in lung lavage fluid during influenza infection. We have also demonstrated that TNFα levels within the lung parenchyma increase during influenza infection and these levels can be lowered significantly by parental treatment with TNFα-neutralizing antibodies. These results indicate that TNFα is released into the airways and parenchyma of the lung in response to influenza A virus infection.

Intranasal inoculation of mouse-adapted influenza virus has been shown by numerous investigators to cause severe lower respiratory disease.[16,20,21] TNFα, administered by aerosol and i.v., has been shown to induce lesions similar to those caused by influenza virus infection.[22,23] Our study demonstrated an association between virus-induced TNFα levels and the development of the pulmonary lesions of influenza pneumonia. We have shown that TNFα-neutralizing antibody treatment and lowered TNFα levels in the lung, reduce lesion development and increase survivorship. These findings support the idea that TNFα mediates pulmonary inflammation and the lesions of influenza pneumonia.

In vitro studies indicate that TNFα can render uninfected cells resistant to viral infection by early inhibition of viral replication.[5,6,24,25] TNFα, alone and in synergy with interferon, has direct cytolytic action against virus-infected cells *in vitro*.[5–7,26,27] The mechanism of cell killing is unclear, although TNFα has been shown to induce both known forms of cell death, apoptosis and necrosis. The increase in TNFα levels in lungs during influenza infection and the *in vitro* antiviral/anticellular action of TNFα against a variety of viruses suggest TNFα may have a positive role in virus clearance from pneumonic lungs. If this were the case, then the reduction of TNFα levels through treatment with TNFα-neutralizing antibodies should result in increased virus titers in the lungs. However, a significant difference between the titers of treated and untreated virus-infected mice was not found in our study, and the peak in TNFα did not correlate with changes in virus titers within the lung. Possible explanations for this finding include: (1) TNFα does not have anti-viral activity against influenza virus *in vivo*, (2) rapid replication of the highly virulent virus strain used in this study obscured any subtle differences in virus titers due to the antiviral actions of TNFα, or (3) alteration of TNFα levels in the lung are compensated for by increased release of other cytokines, such as interferons.

With TNFα, as with other cytokines, there appears to be a fine line between benefit and harm. Early in infection, low levels of TNFα may be helpful in local control of injury and in limiting virus replication. As the infection resolves, TNFα may also be beneficial in the tissue repair process, stimulating the growth of fibroblasts and endothelial cells. If viral replication is not inhibited at an early stage, inflammation

and infection becomes widespread. At this point, high levels of TNFα within the lung may then promote the tissue damage and lung consolidation seen in influenza pneumonia. In this situation, TNFα's actions may be harmful to the host.

Materials and methods

Virus. Influenza virus A/PR/8/34 (H1N1) was obtained as a kind gift from Virginia Hinshaw, University of Wisconsin-Madison. Virus stocks were grown in 10–11 day old embryonated chicken eggs. Allantoic fluid was collected and frozen at −70°C.

Antibody. Rabbit anti-mouse TNFα neutralizing polyclonal serum was obtained from Genzyme Corporation, Boston, MA, U.S.A. One μl of the antiserum neutralizes approximately 1000 Units of mouse TNFα bioactivity in an L929 cell cytotoxicity assay. Antibody neutralization of TNFα bioactivity was confirmed *in vitro* using the L929 cell TNFα bioassay, as reported by Flick and Gifford.[28] Normal rabbit serum (NRS) was from a non-immunized rabbit from the Centralized Biological Laboratory, PSU.

Mice. Eight-week-old, male, Crl:CD-1(ICR)BR (CD-1) outbred mice were obtained from Charles River Laboratories (Wilmington, MA, U.S.A.). They were singly housed in microisolator caging according to NIH and OPR guidelines.

Treatment groups— There were four experimental groups of mice:
1. Control = administered 50 μl PBS intranasally (i.n.) and inoculated 50 μl PBS i.p.
2. Antibody control = 50 μl PBS i.n. and 50 μl antibody i.p.
3. Virus = inoculated 5×10^6 Egg Infectious Dose 50 (EID50) units of virus in 50 μl PBS i.n. and 50 μl PBS i.p.
4. Treatment = 50 μl virus i.n. and 50 μl antibody i.p.

In one experiment, the survival of mice treated with NRS was compared with mice treated with PBS ($n = 10$). There was no difference in survival between these two sham-treated control groups (data not shown).

Survivorship. Treated and sham-treated virus-infected mice were observed for development of clinical signs of disease and for morbidity for 10 days. For humane reasons, mice were enthanized if moribund, i.e. ruffled hair coat, respiratory distress and poorly responsive to external stimuli, to minimize suffering. The same individual made the decision as to when animals were moribund in an attempt to decrease any variation from subjective assessment.

Lung homogenate. Lung tissue samples were placed in sterile PBS (1 ml PBS per 0.1 gram tissue). Lung tissue was disrupted in a Stomacher Lab Blender (Seward Medical, London, U.K.) or by gently teasing tissue through a sterile 60 mesh wire screen. The resulting cell suspension was centrifuged at 2000 rpm for 20 min. The supernatant was decanted and stored at −70°C until assayed.

Titers. Infectious virus titers of lung samples were determined using embryonated chicken egg inoculation and hemagglutination assay (HA), as previously described.[29] Titers were calculated using the method of Reed and Muench[30] and reported in EID50 units.

Morphometry. Histologic evaluation of lungs fixed with 10% buffered formalin was performed using light microscopy. The left lungs from mice (day 5 p.i.) were embedded in paraffin, midsagittally sectioned and the sections stained with hematoxylin and eosin. Sections were scanned to determine the presence and distribution of lesions and the nature of the cellular infiltrate. The percentage of involved lung area and the mean cross-sectional surface area were determined morphometrically,[31] using BioQuant System IV computer software (R&M Biometrics, Inc., TN, U.S.A.) and expressed in square microns. Gross lesions were assessed by visual observation and reported as percent of the lung involved. Inflammation was assessed by light microscopy. Morphometric measurement of hypercellular and total cross-sectional surface area was determined on mid-sagittal sections of the left lung lobe expressed in μm^2.

TNFα measurements. Quantitation of immunologically reactive TNFα, in broncholavage fluid and cell-free lung homogenate, was measured using a commercial ELISA kit (Factor-Test TNF, Genzyme, Corporation, Boston, MA, U.S.A.).

Analysis of data. The data was analyzed using the Minitab software program (Minitab, Inc., State College, PA, U.S.A.) The data from different samples were compared by Students *t*-test and by analysis of variance test. The significance level was present at $\alpha = 0.05$.

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